

**The Design and Synthesis of Novel Heterocyclic
Inhibitors of the DNA-Repair Enzyme,
Poly(ADP-Ribose) Polymerase, as Potential
Resistance-Modifying Agents.**

**A Thesis Submitted to the University of Newcastle upon Tyne
for the Degree of Doctor of Philosophy**

NEWCASTLE UNIVERSITY LIBRARY
097 51556 9
Thesis L6049

Sheila Srinivasan B.Sc. (Hons)

November 1997

For my parents

Declaration

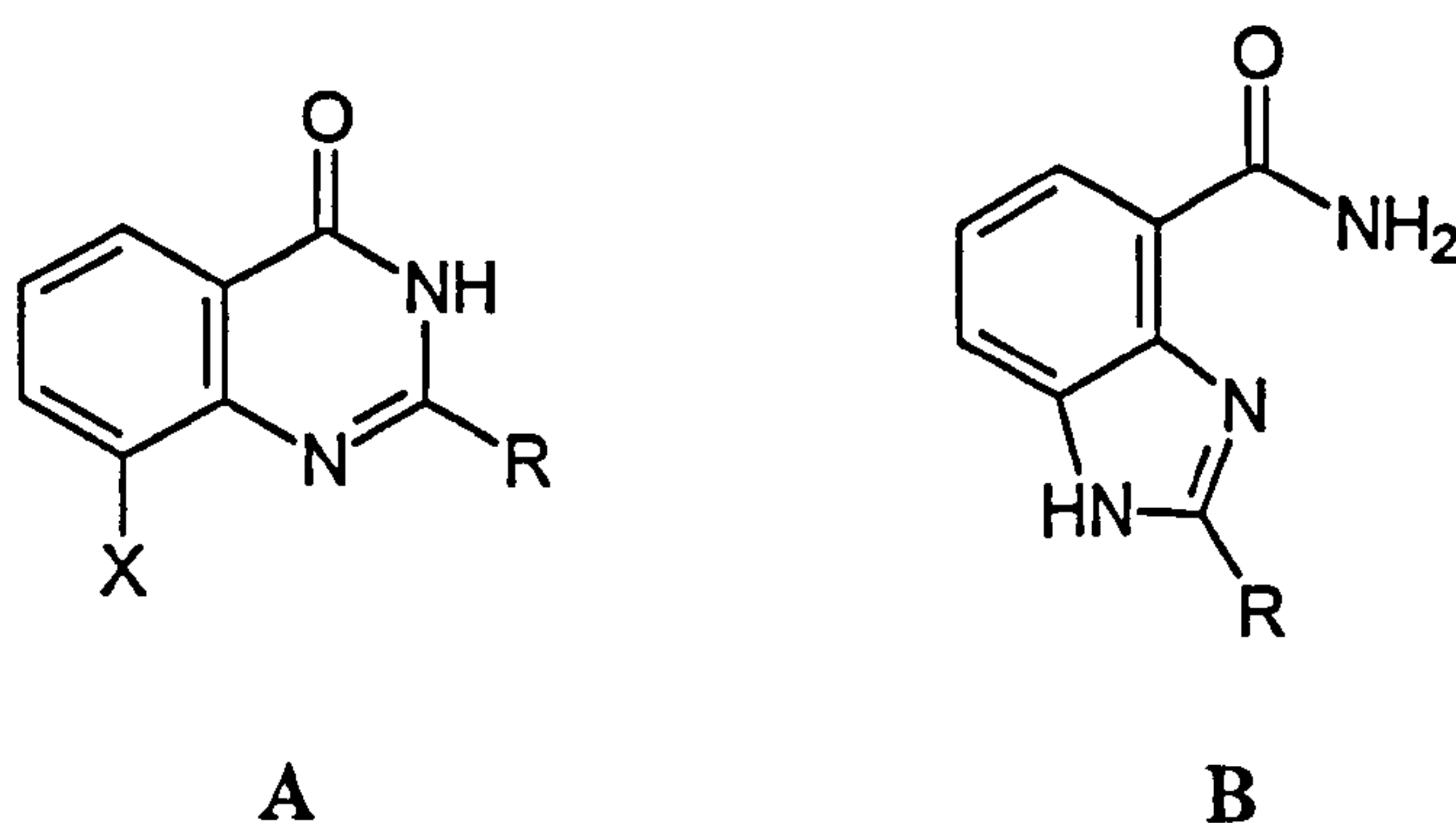
The work described in this thesis was carried out in the Department of Chemistry in collaboration with the Cancer Research Unit at the University of Newcastle upon Tyne between October 1994 and October 1997 and is original except where acknowledged by reference.

No part of this work is being, or has been, submitted for a degree, diploma or any other qualification at any other university.

Abstract

The abundant nuclear enzyme, poly(ADP-ribose) polymerase (PARP) is activated by DNA strand breaks and catalyses the transfer of ADP-ribose moieties from its substrate, nicotinamide adenine dinucleotide (NAD^+), to various histone- or non-histone nuclear acceptor proteins. The net result is the formation of long, homopolymeric chains, the exact purpose of which is not clearly understood. Since this process is thought to facilitate DNA repair, the PARP enzyme represents a possible therapeutic target. A well known mechanism by which tumours become resistant to anticancer treatments is increased DNA repair. Inhibition of PARP may thus be a strategy for the potentiation of DNA-damaging agents and PARP inhibitors may function as resistance-modifying agents in conjunction with cancer chemotherapeutic agents.

The aim of this research was to design and synthesise novel heterocyclic inhibitors of PARP, based on the existing knowledge of structure-activity requirements. A great deal of information has already been gathered from the use of early inhibitors, such as nicotinamide and 3-aminobenzamide (3AB). However, these inhibitors lack potency, specificity for the enzyme, and aqueous solubility, and so are limited in their use as clinical agents.



A novel series of quinazolin-4-[^3H]-ones (structure A) has been synthesised by a high-yielding, reproducible pathway, including derivatives bearing electron-withdrawing and electron-donating substituents in the 2-position. These derivatives exhibit excellent *in vitro* PARP inhibitory activity, with IC_{50} values in the micromolar concentration range,

and the selected compound, NU1025 (8-hydroxy-2-methylquinazolin-4-[3*H*]-one) has been shown to potentiate the effects of a range of mechanistically diverse anticancer agents, including γ -irradiation. A water-soluble phosphate prodrug derivative of NU1025 has been synthesised and this shows promising enzyme-mediated conversion to the parent compound in plasma.

A second series of 1*H*-benzimidazole-4-carboxamides (structure **B**) has been synthesised bearing mono-, di- or trisubstituted aryl rings in the 2-position. Biological evaluation of this series has shown that these derivatives are among the most potent PARP inhibitors reported to date, with K_i values in the low nanomolar concentration range.

Acknowledgements

I would like to thank my supervisors Dr Roger J Griffin and Professor Bernard T Golding for their invaluable help and supervision throughout my PhD. As part of the Anticancer Drug Discovery Initiative (ADDI), members of the Cancer Research Unit, Medical School, University of Newcastle upon Tyne also deserve a mention, as their guidance and biological discussions were both an interesting and integral part of this research project. Thanks go to Dr DR 'Herbie' Newell and Professor A Hilary Calvert, and especially to Dr Nicola J Curtin and Dr Barbara W Durkacz, both of whom showed remarkable patience in explaining biological aspects of this work. Particular thanks go to Miss Karen Bowman, who painstakingly performed the inhibition, potentiation and kinetic studies on selected PARP inhibitors and Dr Jo Calvete, who carried out preliminary HPLC studies on the quinazolinone prodrug.

I would like to thank the following people for technical support: Mr Eddie Hart, Mr Alan Liddle, Mr John Marshall and Mr Richard Davison, who has not only been a good friend but an excellent technical assistant. Thanks also go to Mrs Linda Cook, Dr MN Stuart Hill and Dr Nick H Rees for providing an excellent NMR service, Mr Steve Addison for mass spectrometry and Mr Dave Dunbar for elemental analysis and infrared spectroscopy.

Thanks go to Miss Hannah C Mullen and Mr Richard Davison for the syntheses of 8-methoxy-2-(4'-azidophenyl)quinazolin-4-[3H]-one and 8-methyl-2-(4'-azidophenyl)quinazolin-4-[3H]-one, respectively.

Many thanks to the members of Lab 3.16, especially Alex, Liz, Hannah, Sharon, Claire, Martin and Julian, and to all my friends, both chemists and non-chemists, for their friendship and support over the last six years.

I would like to thank my parents, both of whom have been a great source of encouragement throughout my time at university, my sister Sujay, for her continued support and Bill Bonney for his patience in general!

Finally, I gratefully acknowledge the North of England Cancer Research Campaign (NECRC) and Cancer Research Campaign Technology (CRCT) for financial support.

Contents

<i>Declaration</i>	<i>i</i>
<i>Abstract</i>	<i>ii</i>
<i>Acknowledgments</i>	<i>iv</i>
<i>Contents</i>	<i>v</i>
<i>Abbreviations</i>	<i>x</i>

Chapter One: Introduction

1.1	DNA Repair	1
1.1.1	DNA Excision Repair	2
1.1.2	UV Radiation-induced DNA Damage	3
1.1.3	Mismatch Repair	4
1.1.4	Alkylation-induced DNA Damage	5
1.2	Drug Resistance	6
1.2.1	Mechanisms of Resistance	7
1.2.2	Multidrug Resistance	10

Chapter Two: Poly(ADP-ribose) Polymerase (PARP)

2.1	ADP-Ribosylation Reactions	12
2.1.1	Mono(ADP-ribosyl)ation Reactions	13
2.1.2	Cyclic ADP-ribose	14
2.2	Poly(ADP-ribose) Polymerase : PARP [EC 2.4.2.30]	16
2.2.1	Poly(ADP-ribosyl)ation	16
2.3	Structural Conservation of PARP Between Species	17
2.4	Modular Organisation of PARP	19
2.4.1	The DNA-Binding Domain (DBD)	20
2.4.1.1	Activation of PARP by DNA Strand Breaks	23
2.4.2	The Automodification Domain	24
2.4.3	The Catalytic Domain	25

2.5	Catalytic Activities of PARP	26
2.5.1	Automodification	26
2.5.2	Heteromodification	31
2.6	Catabolism of Poly(ADP-ribose)	32
2.6.1	Poly(ADP-ribose) Glycohydrolase	33
2.6.2	ADP-ribosyl Protein Lyase	34
2.7	Distribution of Poly(ADP-ribose) Metabolising Enzymes Within the Cell	34
2.7.1	Detection of Poly(ADP-ribose)	35
2.8	Summary : The Poly(ADP-ribosyl)ation System	37

Chapter Three: The Involvement of PARP in Cellular Processes

3.1	Methods for Studying PARP	40
3.1.1	The Use of Chemical Inhibitors	40
3.1.2	Molecular Genetic Approaches	40
3.2	Proposals for the Role of PARP	41
3.2.1	Modulation of Chromatin Structure by Poly(ADP-ribosyl)ation	41
3.2.2	Poly(ADP-ribosyl)ation of Nuclear Proteins	42
3.2.3	PARP, Chromosome Aberrations and Sister Chromatid Exchange (SCE)	44
3.2.4	Poly(ADP-ribosyl)ation and Gene Amplification	44
3.2.5	Poly(ADP-ribosyl)ation and DNA Repair	45
3.2.5.1	Histone Shuttle Mechanism	45
3.2.5.2	Non-Histone Mechanism	48
3.2.5.3	Poly(ADP-ribosyl)ation and Ligation of DNA	50
3.2.5.4	PARP and Excision Repair	52
3.2.6	PARP and DNA Replication	54
3.2.7	PARP and Cell Death	55
3.2.8	Poly(ADP-ribosyl)ation and the Cell-Cycle Checkpoint	57
3.2.9	Poly(ADP-ribose) Activity and HIV-1 Infection	57
3.3	The Use of Transgenic Model Systems	58
3.3.1	The Development of PARP 'Knockout' Mice	58

3.4	PARP and Carcinogenesis	59
3.4.1	The Relevance of PARP Inhibitors as Clinical Agents	60
3.5	Summary	60

Chapter Four: The Development of Chemical Inhibitors of PARP

4.1	Introduction	63
4.2	Development of PARP Inhibitors	63
4.2.1	Nicotinamide and Benzamide Analogues	64
4.2.2	Specificity of PARP Inhibitors	68
4.2.3	Mechanism Based PARP Inhibitors	68
4.2.4	Conformationally Restricted Inhibitors of PARP	70
4.2.5	Nucleoside Analogues of Thymidine as PARP Inhibitors	73
4.2.6	Design and Synthesis of Heterocyclic PARP Inhibitors	74
4.3	Summary of Structure-Activity Requirements	75
4.4	The Structure of the Enzyme Active-Site	76

Chapter Five: Development of the Quinazolinone Series

5.1	Quinazolinones as Pharmaceutical Agents	79
5.2	General Methods of Quinazolinone Synthesis	80
5.3	Quinazolinones as PARP Inhibitors	81
5.3.1	Rearrangement of Benzoxazole-4-carboxamides to 8-Hydroxyquinazolin-4-[3H]-ones	82
5.3.2	Quinazolinone Structure-Activity Relationships	84
5.4	Aims of Quinazolinone Research	84
5.5	Syntheses of Quinazolinone PARP Inhibitors	85
5.5.1	Synthesis of 2-Alkyl Quinazolinones	85
5.5.2	Synthesis of 2-Aryl Quinazolinones	89
5.5.3	Synthesis of 2-(4'-Substituted-phenyl) Quinazolinones	91
5.5.3.1	Synthesis of Quinazolinones Bearing Electron-Withdrawing Substituents	91

5.5.3.2	Synthesis of Quinazolinones Bearing Electron-Donating Substituents	99
5.6	Synthesis of Control Compounds	101
5.7	Limitations of Quinazolinone Synthesis	102
5.8	Design of Quinazolinone Prodrugs	103
5.8.1	Synthesis of Carbamate Ester Prodrugs	104
5.8.2	Synthesis of Phosphate Prodrugs	106
5.9	Summary	112

Chapter Six: Development of the Benzimidazole Series

6.1	Benzimidazoles as Pharmaceutical Agents	113
6.2	General Methods of Benzimidazole Synthesis	114
6.3	Benzimidazoles as PARP Inhibitors	116
6.3.1	Benzimidazole Structure-Activity Relationships	116
6.3.2	Proposed Synthetic Route to Benzimidazole-4-Carboxamides	117
6.4	Aims of Benzimidazole Research	119
6.5	Syntheses of Benzimidazole PARP Inhibitors	120
6.5.1	Limitations of the Proposed Synthetic Route	120
6.5.2	Modifications to the Proposed Synthetic Route	122
6.5.3	Synthesis of Benzimidazoles from Diacylated Precursors	123
6.5.4	Synthesis of Benzimidazoles from Isatin Derivatives	124
6.5.5	Copper-catalysed Benzimidazole Synthesis	127
6.6	Synthesis of Benzimidazole Phosphate Prodrugs	131
6.7	Summary	132

Chapter Seven: Biological Results and Discussion

7.1	Introduction	133
7.2	Inhibition Studies with PARP Inhibitors	133
7.2.1	Permeabilised Cell Assay	133
7.2.2	96-Well Plate Assay	134

7.3	Results of Inhibition Studies with Quinazolinones	134
7.4	Evaluation of the Quinazolinone Prodrugs	139
7.4.1	Preliminary Results from <i>In Vivo</i> studies	140
7.5	Results from Potentiation Studies	143
7.5.1	Potentiation of Alkylating Agents	143
7.5.2	Potentiation of Ionising Radiation	144
7.5.3	Potentiation of Other DNA-Damaging Agents	146
7.5.3.1	PARP Inhibitors and Bleomycin	146
7.5.3.2	PARP Inhibitors and Antimetabolites	146
7.5.3.3	PARP and Topoisomerase Inhibitors	146
7.6	Results of Inhibition Studies with Benzimidazoles	149
7.7	Results from Kinetic Experiments	152
7.8	Summary	153

Chapter Eight: Experimental

8.1	Index of Compounds Synthesised	154
8.2	General Experimental Details	157
8.3	Standard Procedures	158
8.4	Quinazolinone Experimental Procedure	162
8.5	Benzimidazole Experimental Procedure	189

Chapter Nine: References	208
---------------------------------	------------

Abbreviations

A	α	alpha
	Å	Angstrom
	3AB	3-Aminobenzamide
	ADDI	Anticancer Drug Discovery Initiative
	Ade	Adenosine
	ADP	Adenosine diphosphate
	AIDS	Acquired immunodeficiency syndrome
	5'-AMP	5'-Adenosine monophosphate
	Anal.	Analysis
	AP	Apurinic/apyrimidinic
	aq.	aqueous
	Ar	aryl
	Arg	Arginine
	Asp	Aspartate
	atm	atmosphere
	ATP	Adenosine triphosphate
B	β	beta
	BER	Base excision repair
	Bn	benzyl
	br	broad
C	^{13}C	Carbon 13 isotope
	cADPR	Cyclic adenosine diphosphate ribose
	cDNA	copy Deoxyribonucleic acid
	conc.	concentrated
	Cys/C	Cysteine

D	d	doublet
	dd	double doublet
	DBD	DNA-binding domain
	DCM	Dichloromethane
	DHFR	Dihydrofolate reductase
	DMAP	<i>N,N</i> -dimethylaminopyridine
	DMF	Dimethylformamide
	DNA	Deoxyribonucleic acid
	DMSO	Dimethyl sulfoxide
E	EI	Electron impact
	EM	Electron microscopy
	ES	Embryonic stem
	Et	Ethyl
F	FAB	Fast atom bombardment
G	Glu	Glutamate
	Gly	Glycine
	GDH	Glutamate dehydrogenase
H	His/H	Histidine
	HIV	Human immunodeficiency virus
	HMG	High mobility group
	HPLC	High performance liquid chromatography
	Hz	Hertz
I	IC ₅₀	Inhibition coefficient value
	ICE	Interleukin-1 β -converting enzyme

K	K _i	Inhibition constant
	kDa	Kilodaltons
L	L/G DH	leucine/glutamate dehydrogenase
	Lys	lysine
M	m	multiplet
	M ⁺	Molecular ion
	<i>m</i> CPBA	<i>meta</i> -Chloroperbenzoic acid
	MDR	multidrug resistance
	MGMT	<i>O</i> ⁶ -methylguanine DNA methyltransferase
	MNNG	<i>N</i> -methyl- <i>N'</i> -nitro- <i>N</i> -nitrosoguanidine
	MNU	<i>N</i> -methyl- <i>N</i> -nitrosourea
	mol.	molar
	mRNA	messenger Ribonucleic acid
	mRNP	messenger Ribonucleoprotein
	MTIC	5-(3-methyl-1-triazino)imidazole-4-carboxamide
N	NAD ⁺	Nicotinamide adenine dinucleotide
	NER	Nucleotide excision repair
	NMR	Nuclear magnetic resonance
	NLS	Nuclear localisation signal
	Nic	Nicotinamide
	NBD	NAD ⁺ -binding domain
	NCS	<i>N</i> -Chlorosuccinimide
P	P	Phosphate
	PARG	Poly(ADP-ribose) glycohydrolase
	PARP	Poly(ADP-ribose) polymerase
	PARP-CF	Poly(ADP-ribose) polymerase-catalytic fragment
	Ph	phenyl

	P _i	Inorganic phosphate
	PPA	Polyphosphoric acid
	ppm	parts per million
	Pr	propyl
	PR-AMP	2'-(or 3')-(5''phosphoribosyl)-5'-adenosine monophosphate
R	RNA	Ribonucleic acid
	rib	ribose
S	s	singlet
	SAM	<i>S</i> -adenosyl methionine
	SCE	Sister chromatid exchange
	Ser	Serine
	SV40	Simian virus 40
T	TCA	Trichloroacetic acid
	THF	Tetrahydrofuran
	TLC	Thin layer chromatography
	TMS	Tetramethylsilane
	Trp	Tryptophan
	TS	Thymidylate synthase
	Tyr	Tyrosine
U	UDS	Unscheduled DNA synthesis
	UV	Ultraviolet light

Chapter One: Introduction

1.1 DNA Repair

The damage continually being inflicted upon DNA, by environmental and endogenous genotoxic agents, poses a severe threat to the all important maintenance of genetic integrity. Although cells may be equipped with a variety of intricate mechanisms to enable them to repair or cope with numerous types of DNA damage, the consequences of defective DNA repair with respect to human health, may manifest themselves as forms of cancer, developmental defects or neurological abnormalities.¹

A classical method for investigating any biochemical system involves the inhibition of specific steps in a given sequence of reactions and then subsequent evaluation of the consequences to the system.² However, the complexity in the structural organisation of chromatin and its many interactions with component enzymes renders the analyses of metabolic processes associated with DNA extremely challenging. In recent years, the use of rodent cell mutants for gene cloning and biochemical studies has significantly improved the understanding of mammalian repair processes.

DNA repair is generally considered to involve the removal or bypass of damage-induced lesions, although DNA undergoes a whole range of transformations during normal growth and development. For example, DNA rearrangements, splicing of sequences and rejoining of strand breaks are just some of the ways that DNA is manipulated. Thus, it would be reasonable to suppose that biochemical processes and enzymes involved in such manipulation of DNA could also be related to the management of DNA damage, or might be induced by damage or inhibition of DNA synthesis.³

Spontaneous Breakdown of DNA

DNA, incubated in physiological solutions (pH 7.4, 37 °C), shows spontaneous breakdown over several days.³ This breakdown is characterised by the loss and

deamination of bases (*e.g.* cytosine is deaminated to uracil and adenine to hypoxanthine). It has been estimated that the number of spontaneous lesions generated per human cell per day is around 7000.⁴

Other Types of DNA Damage

Damaged DNA can also result from exposure to exogenous factors, such as UV and ionising radiation, or treatment with electrophilic chemicals. Extensive damage from the cytoplasmic surroundings may also be inflicted upon DNA, for example, through exposure to reactive metabolites, which may cause oxidative damage or alkylation of DNA.⁵

Since continual DNA damage would amount to a high rate of mutation during genomic replication, it is not surprising that many cells have evolved complex but efficient methods to counteract potentially lethal damage. The following section describes some of the more clearly defined mechanisms for DNA damage repair.

1.1.1 DNA Excision Repair

DNA excision repair is comprised of two pathways: nucleotide excision repair (NER) and base excision repair (BER).

Nucleotide Excision Repair (NER)

NER is the most complicated of the excision repair processes. Several studies have shown that a deficiency in NER in humans is associated with the rare but life-threatening genetic disorders of xeroderma pigmentosum, trichothiodystrophy and Cockayne syndrome,⁶ underlying the importance of NER for the maintenance of genomic integrity.

The process of NER removes damaged DNA as part of an oligonucleotide fragment. The intact strand of DNA serves as a template for the new DNA, which is then used to replace the removed fragment. NER involves the products of about 30 genes and can recognise a broad variety of chemical alterations in DNA. A great deal of progress has been made in

the study of NER, and it is likely that in the next few years, the connections between clinical phenotypes and molecular defects will be more clearly understood.⁶

Base Excision Repair (BER)

The BER pathway has evolved as a cellular protection mechanism, to guard against the deleterious effects of endogenous oxidative DNA damage. However, some exogenous DNA-damaging agents, such as ionising radiation, result in hydroxyl radicals being formed which can be as detrimental as the direct formation of radicals in DNA. BER is also important in the repair of spontaneous DNA damage and damage induced by certain endogenous factors.⁵

The main enzymes involved in BER are DNA glycosylases. These enzymes catalyse the cleavage of the *N*-glycosylic bond between a damaged or modified base and the deoxyribose group of the nucleotide residue. The resulting site is known as an apurinic/apyrimidinic (AP)-site, which is then removed by an (AP)-endonuclease or an (AP)-lyase. The appropriate phosphodiesterase removes the remaining deoxyribose phosphate residue and the gap is filled by a DNA polymerase and sealed by a DNA ligase.⁵

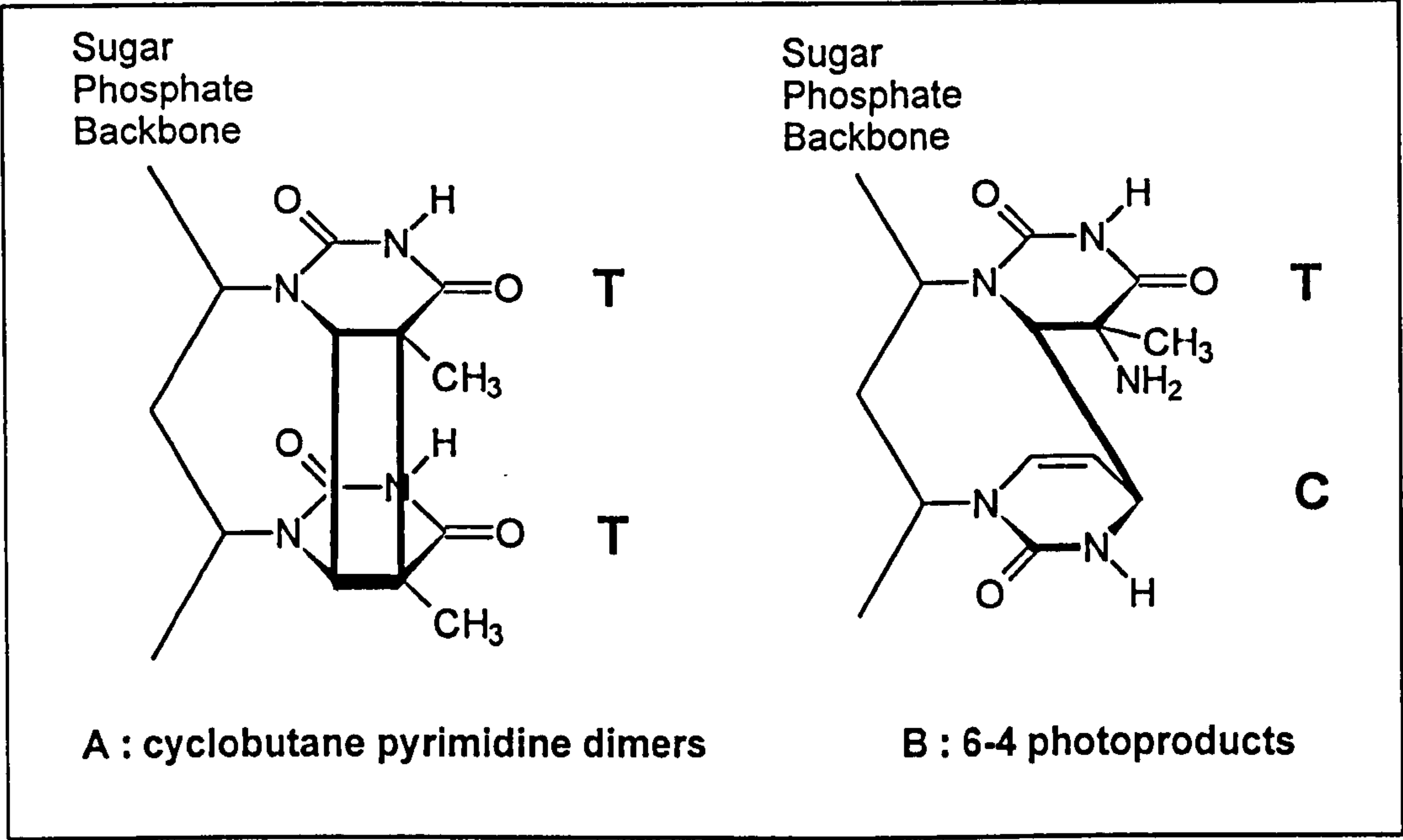
The BER process has been much less extensively studied in DNA repair enzymology compared to the NER pathway. Since there is no human or animal model which is defective in its BER pathway, the significance of BER in protection against mutational disorders has proved difficult to determine.⁵

1.1.2 UV Radiation-induced DNA Damage

Exposure to UV radiation can result in cell death, mutation and neoplastic transformation, primarily due to the formation of UV radiation-induced DNA photoproducts.⁷ The first and second most frequently occurring UV photoproducts are cyclobutane pyrimidine dimers and 6-4 photoproducts, respectively (see *Figure 1.1.2*).⁷

Figure 1.1.2 The major UV light-induced DNA photoproducts:⁷

A : cyclobutane pyrimidine dimers **B : 6-4 photoproducts**



In many species, there may be more than one operative pathway involved in the repair of major toxic and mutagenic UV photoproducts. In humans and most other organisms, the pathway for the removal of cyclobutane pyrimidine dimers is thought to be NER.⁸ Some organisms are able to excise cyclobutane pyrimidine dimers by way of an additional BER pathway, although BER is not generally regarded as the method for removal of UV photoproducts.⁷

1.1.3 Mismatch Repair

DNA mismatch repair systems have been extensively studied and genetic defects in mismatch repair genes have been associated with cancer susceptibility.⁹ Indeed, mismatch-repair-defective cell lines have been identified from a variety of tumour types including colon, endometrial, ovarian, prostate and uterine tumours.⁹

The mismatch repair pathway involves the repair of mispaired bases, which may be formed as a result of exposure to DNA-damaging agents or during DNA replication and

genetic recombination. Mismatch repair is also thought to be involved in other vital processes, such as certain types of NER and the cell-cycle check-point system.⁹

1.1.4 Alkylation-induced DNA Damage

Alkylating agents cause damage to DNA by reacting with its nucleophilic sites. The net result is the formation of alkyl-DNA adducts, which can have mutagenic consequences. Exposure of mammalian cells to methylating or ethylating agents gives rise to alkylation of guanine at the O^6 -position.¹⁰ Methylation or ethylation of the O^6 -position of guanine produces a miscoding lesion, which may result in the incorporation of a guanine-thymidine base-pair, instead of a guanine-cytosine pair, into DNA during replication.¹¹ The DNA repair protein O^6 -methylguanine DNA methyltransferase (MGMT) is responsible for the removal of DNA-alkyl groups and stoichiometrically transfers the O^6 -methyl group from the guanine to a cysteine residue.³ Thus, MGMT has evolved as a cellular protection mechanism against the accumulation of pre-toxic alkylation lesions in normal, healthy cells. Since the strict definition of an enzyme states that it should not be consumed during the course of the reaction, MGMT resembles a 'suicide' enzyme, in that it is not regenerated after repair of the lesion.

In certain human tumours and tumour cell lines, MGMT no longer appears active.⁴ Tumour cell lines lacking the repair activity (known as mer- or mex- cells) are particularly sensitive to simple alkylating agents and cells possessing repair activity (mer+ or mex+ cells) are generally resistant to such drugs.

The ubiquitous distribution of MGMT in normal cells infers the presence of endogenous DNA methylation damage. *S*-adenosylmethionine (SAM) is responsible for the shuttling of methyl groups around the cell during the course of normal biosynthetic reactions and has been identified as a candidate for carrying out endogenous DNA methylation.¹² The reaction involves non-enzymatic methyl group transfer but the mechanism of donation to an oxygen would occur only slowly and therefore, would be unlikely to produce a significant amount of O^6 -methylguanine. Swann *et al* reasoned that transfer to a sulfur

atom would be easier than to oxygen.¹³ They replaced the O^6 -atom of O^6 -methylguanine with sulfur to give 6-thioguanine, and demonstrated that the transfer was indeed, several 1000-fold faster to the S^6 -position than to the O^6 -position. The facile methylation of 6-thioguanine has been suggested to represent the mechanism of cytotoxicity of this agent.¹³ 6-thioguanine is used for the treatment of acute leukaemia. It is metabolised by the pathway that recycles purines and is subsequently incorporated into DNA.¹⁴ Methylation tolerant cells generally develop cross-resistance and have the ability to replicate 6-thioguanine-containing DNA without detrimental effects.¹⁵

Thus, although all of the above repair processes are absolutely critical for ensuring the survival of normal cells in an error-free way, the consequences of the repair mechanisms present in tumour cells must also to be considered. Since many cancer chemotherapeutic agents induce DNA damage as a means of destroying transformed cells, repair of such damage by tumour cells may impair the efficacy of antitumour drugs. Drug resistance is a major obstacle in cancer chemotherapy and investigations into the mechanisms of resistance have been the focus of extensive study over the last two decades. The following section gives a brief overview of drug resistance and some of the molecular mechanisms involved.

1.2 Drug Resistance

Drug resistance has emerged as one of the most important therapeutic problems in medical oncology. The identification of new drugs with activity against resistant tumours continues to challenge the pharmaceutical industry, largely because the effectiveness of chemotherapy is compromised by the development of resistance.

Drug resistance can be categorised as either intrinsic or acquired, and can develop to a single drug or, simultaneously, to a variety of agents.¹⁶ The distinction is derived from whether the tumour is resistant from the onset of treatment (intrinsic) or whether the tumour responds initially but then gradually becomes resistant during the course of treatment (acquired). An increasing body of evidence suggests that mixtures of drug

resistance patterns are now emerging in human tumours. It has been estimated that drug resistance in general contributes to more than 90% of all cancer fatalities.¹⁶

Intrinsic Resistance

Examples of intrinsically resistant tumours include those of the respiratory system, the digestive organs, the urinary organs, the brain or the central nervous system.¹⁶ Intrinsically resistant tumours may often arise from duct cells or cells which line excretory organs.¹⁷ These types of cells would normally function in the detoxification, transport and elimination of compounds of a toxic nature. However, if these cells were to retain their normal functions in malignant tumours, they may then detoxify and eliminate chemotherapeutic drugs.¹⁶

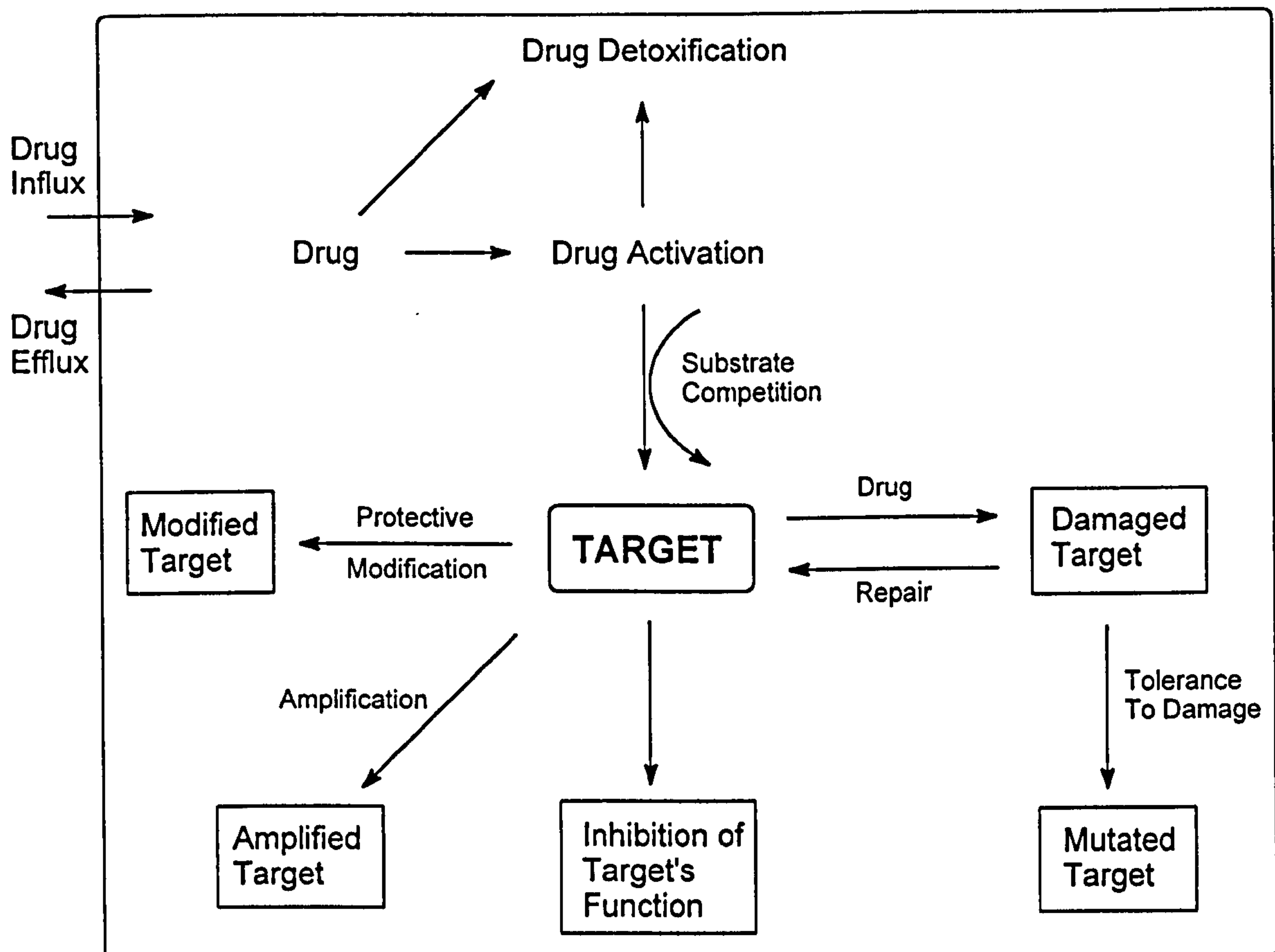
Acquired Resistance

Acquired resistance refers to the emergence of a resistant strain or cell-line, from a population that was initially drug-sensitive. This form of resistance may arise through genetic alterations within the tumour cells. However, it is believed that there may be several different mechanisms involved, which in some cases may operate simultaneously.¹⁷

1.2.1 Mechanisms of Resistance

Figure 1.2.1 illustrates some of the many biochemical mechanisms of resistance (see main text for details).¹⁷ Chemotherapeutic resistance has been studied in great detail and specific mechanisms identified in human tumour cell lines include defective drug transport, enhanced detoxification mechanisms and altered DNA repair (e.g. increased repair capacity or increased tolerance to damage).¹⁶ However, resistance to any one particular drug may occur *via* more than one mechanism. Resistance to a range of compounds, either structurally related or unrelated, is frequently observed (referred to as multidrug, or pleiotropic, resistance).¹⁶

Figure 1.2.1 Biochemical mechanisms of drug resistance

**Altered Drug Transport**

Effective delivery of a drug to its target site is of crucial importance for maximum drug efficacy. An example of an area which is difficult to target with chemotherapy is the brain. Delivering drugs across the blood-brain-barrier is a major obstacle in therapeutic treatment, and the brain is often referred to as a 'sanctuary site' for tumour cells.^{17,18} Altered drug transport is an example of a mechanism dependent on factors which are essentially unrelated to the target cell.

Reduced Drug Uptake

A general mechanism of resistance occurs due to the defective transport of therapeutic agents, and involves drugs which are absorbed passively as well as those which are actively transported into the cell.¹⁷ Many anticancer drugs (e.g. 5-fluorouracil, nitrogen mustard and melphalan) enter cells *via* active transport mechanisms, and mutations which affect these transport processes have been implicated in the resistance of tumour cells to these particular drugs.¹⁷

Increased Drug Efflux

Resistance to certain compounds arises from alterations in the roles of proteins involved in drug efflux. P-glycoprotein is a trans-membrane, energy-dependent, efflux protein and is thought to confer resistance to a variety of anticancer drugs in human tumours¹⁹ (discussed under Section 1.2.2)

Altered Drug Metabolism

Drug-metabolising enzymes play an important role in the regulation of the intracellular concentration of a drug. For example, there may be a decrease in the expression of drug-activating enzymes or an increase in the expression of detoxification enzymes, both of which can lead to resistance. It appears that in some model systems, phase I and phase II enzymes (oxidation and conjugation, respectively) play an important role in cell protection against drug-induced damage.¹⁷

Target Site Alterations

In some cases, drug resistance may arise as a result of a structural change in the target enzyme or protein, or may reflect the complete absence of a specific receptor. For example, a well-known mechanism for hormone-dependent breast cancer results from the lack of the oestrogen receptor.²⁰ Resistance may also occur due to a change in the intracellular concentration of the target site. So, although the target site structure remains unaffected, there may be an increase in target site abundance. An example of a target site alteration is an increase in the levels of the mammalian enzyme dihydrofolate reductase (DHFR) in certain tumour cell lines.²¹ DHFR is involved in the *de novo* synthesis of

purines and thymidine and in a large number of cases, a direct correlation between the level of intracellular DHFR and the degree of resistance to antifolates has been demonstrated.²¹

Repair of Drug-induced Damage

Increased repair of cellular damage is a significant mechanism of drug resistance to alkylating agents and radiation.^{17,18} In most cases, inhibition of the repair is the logical strategy for reversal of the drug-induced resistance. MGMT (Section 1.1.4) has been identified as a factor involved in drug resistance, and can provide protection for tumour cells against alkylating agents by carrying out its normal function of DNA repair.²²

Tolerance to DNA Damage

Some cell lines are able to survive, despite the presence of high levels of DNA damage: for example, cells resistant to alkylating agents can incorporate 6-thioguanine into DNA without harmful effects. However, the mechanistic details of this tolerance and the overall contribution to resistance remain largely unknown, although it is possible that the mismatch repair system is involved.¹⁸

1.2.2 Multidrug Resistance

Multidrug resistance (MDR) is characterised by the simultaneous expression of cellular resistance by tumour cells to a broad variety of structurally and functionally unrelated drugs.¹⁸ MDR has been largely associated with the activities of the membrane protein, P-glycoprotein, as its levels are increased in certain tumour cell lines. However, a variety of antitumour agents have been shown to directly induce P-glycoprotein expression²⁴⁻²⁶ and the presence of increased levels of P-glycoprotein has been correlated with poor responses to chemotherapy. This raises the interesting possibility that the therapy itself could contribute to the development of MDR.

MRP-Associated Multidrug Resistance

Not all cells classified with the MDR phenotype contain increased levels of P-glycoprotein. A second MDR-associated transporter protein was discovered by Cole and colleagues in 1992.²⁷ The protein, identified as MRP, a 190 kDa ATP-binding membrane glycoprotein, appears to be a pump similar to P-glycoprotein, which effluxes drugs from the cell.²³ As with P-glycoprotein MDR, high levels of MRP expression are associated with resistance to anticancer agents.²³

Despite the grave nature of the problem of drug resistance, there is a good deal of hope for the development of novel strategies and combination therapies. By using drugs which target the mechanism of resistance in combination with existing anticancer agents, it may be possible to circumvent the problems of drug resistance and thus restore the desired effects of treatment.

Selective Toxicity

It has been stated that the natural resistance of human tumours to currently used chemotherapeutic agents is attributed to the non-selectivity of these drugs.²⁸ Since cancer cells arise from normal cells, it has proved difficult to identify a target unique to the cancer cell, not present in normal human cells. Most of the currently used anticancer agents employed in chemotherapy have resulted from serendipity, screening and modification of current active agents. A high proportion of cancer chemotherapeutic agents are developed according to knowledge of biochemical pathways. However, it is still abundantly clear that more specific therapeutic agents which exhibit selective toxicity towards cancer cells are urgently required for use in the clinic.

Chapter Two: Poly(ADP-ribose) Polymerase (PARP)

2.1 ADP-Ribosylation Reactions

ADP-ribosylation reactions constitute a group of posttranslational modifications of protein structures using nicotinamide adenine dinucleotide (NAD^+), the most abundant of the respiratory coenzymes, as a donor of the modifying group.²⁹

The study of ADP-ribosylation reactions (or ADP-ribose transfer reactions) is generally considered to have originated in the 1960's, with the discoveries of two distinct processes: mono- and poly(ADP-ribosyl)ation. The first report on the discovery of a polymeric product of an ADP-ribosylation reaction was documented in 1963 by Chambon, Weil and Mandel.³⁰ Data relating to the nature of this novel polymer, known as poly(ADP-ribose), and the enzyme responsible for its synthesis, poly(ADP-ribose) polymerase [PARP: EC 2.4.2.30], were later reported in 1966.³¹ The discovery of mono(ADP-ribosyl)ation reactions was initiated by the findings of Collier and Pappenheimer in 1964, who demonstrated that NAD^+ was absolutely necessary for the inhibition of protein synthesis in cell-free systems by diphtheria toxin.³²

In recent years, with the development of molecular and genetic techniques, significant advances in the understanding of ADP-ribosylation reactions have been made. Both mono- and poly(ADP-ribose) are generally viewed to be involved in signal transduction processes: the eukaryotic mono(ADP-ribosyl) transferases being involved in transducing signals from extracellular components, and poly(ADP-ribosyl)ation being associated with the nuclear compartment.³³

Ten years ago, Lee and coworkers³⁴ reported the discovery of a new metabolite of NAD^+ that was an effective calcium-mobilising agent in sea urchin microsomes. They later confirmed that the metabolite was a cyclic form of ADP-ribose³⁵ and since its discovery,

the amount of information regarding the role of this cyclic NAD^+ metabolite has steadily increased.

The following sections briefly describe important aspects of mono(ADP-ribosyl)ation reactions and cyclic (ADP-ribose) metabolism. Since this thesis is primarily concerned with the process of poly(ADP-ribosyl)ation, information relating to this category of ADP-ribosylation reactions will subsequently be presented in much greater detail.

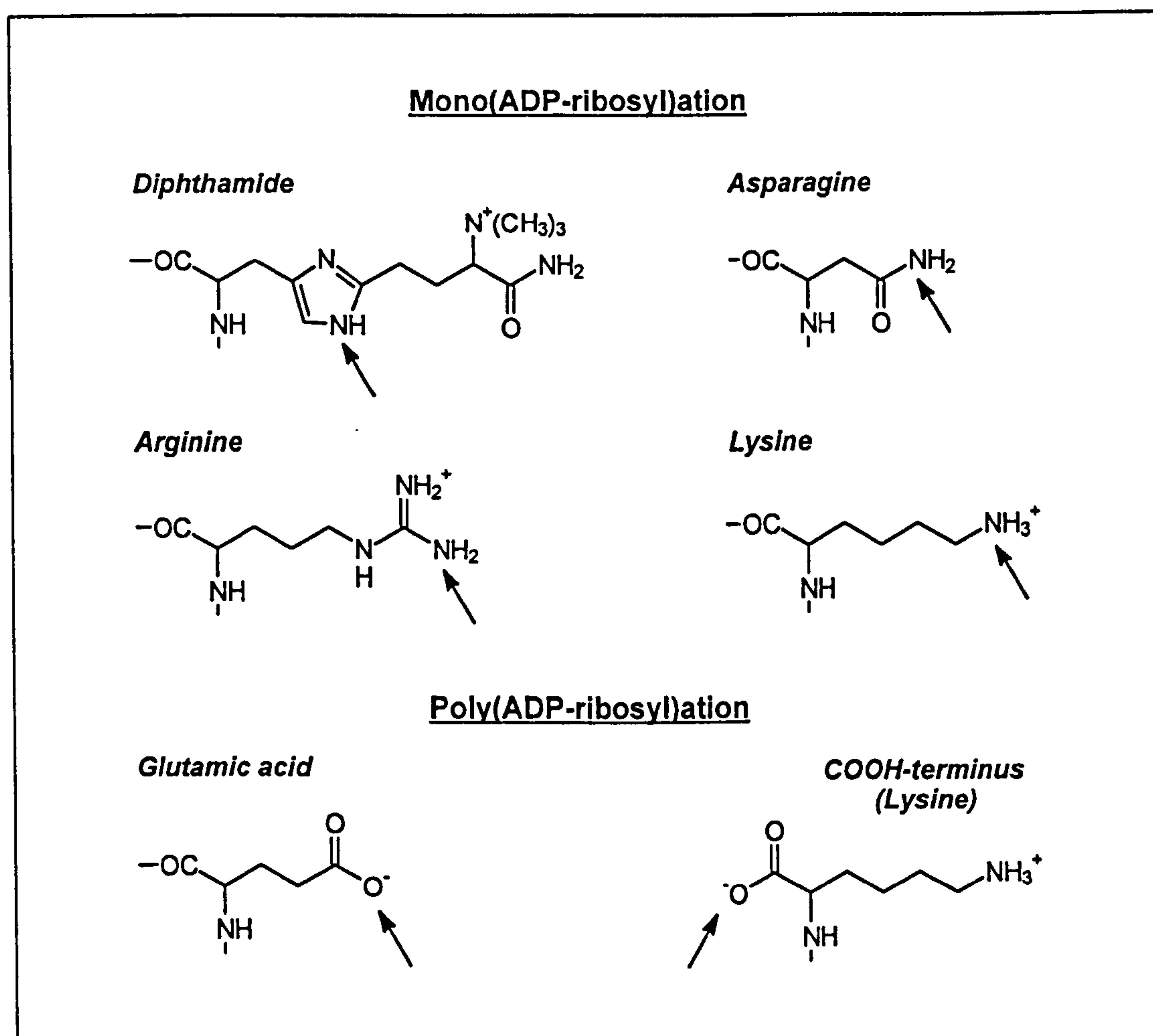
2.1.1 Mono(ADP-ribosyl)ation Reactions

Mono(ADP-ribosyl)ation reactions involve the addition of a single ADP-ribose unit from NAD^+ to an acceptor amino acid or protein molecule, and occur in the cytoplasm and cell membranes of both prokaryotic and eukaryotic cells.²⁹

Several bacterial toxins are classified as mono(ADP-ribosyl) transferase enzymes with targets in eukaryotic cells. These enzymes exhibit remarkable specificity for their substrates, with respect to particular amino acids and proteins. According to this specificity, mono(ADP-ribosyl)ation reactions can be divided into four subtypes: diphthamide-, arginine-, asparagine- and lysine-specific ADP-ribosylations.²⁹

Figure 2.1.1 shows a comparison of the amino acid acceptors for mono(ADP-ribosyl)ation and poly(ADP-ribosyl)ation.²⁹ The arrows indicate the chemical nature of the ADP-ribosyl protein bond formed: mono(ADP-ribosyl)ation results in *N*-glycosidic linkages whereas poly(ADP-ribosyl)ation produces *O*-glycosides.

Mono(ADP-ribosyl)ation of proteins has been shown to affect the activities of proteins involved in crucial metabolic or regulatory pathways,³⁶ such as signal transduction,³⁷ thus, indicating the importance of mono(ADP-ribosyl)ation reactions in biological systems.

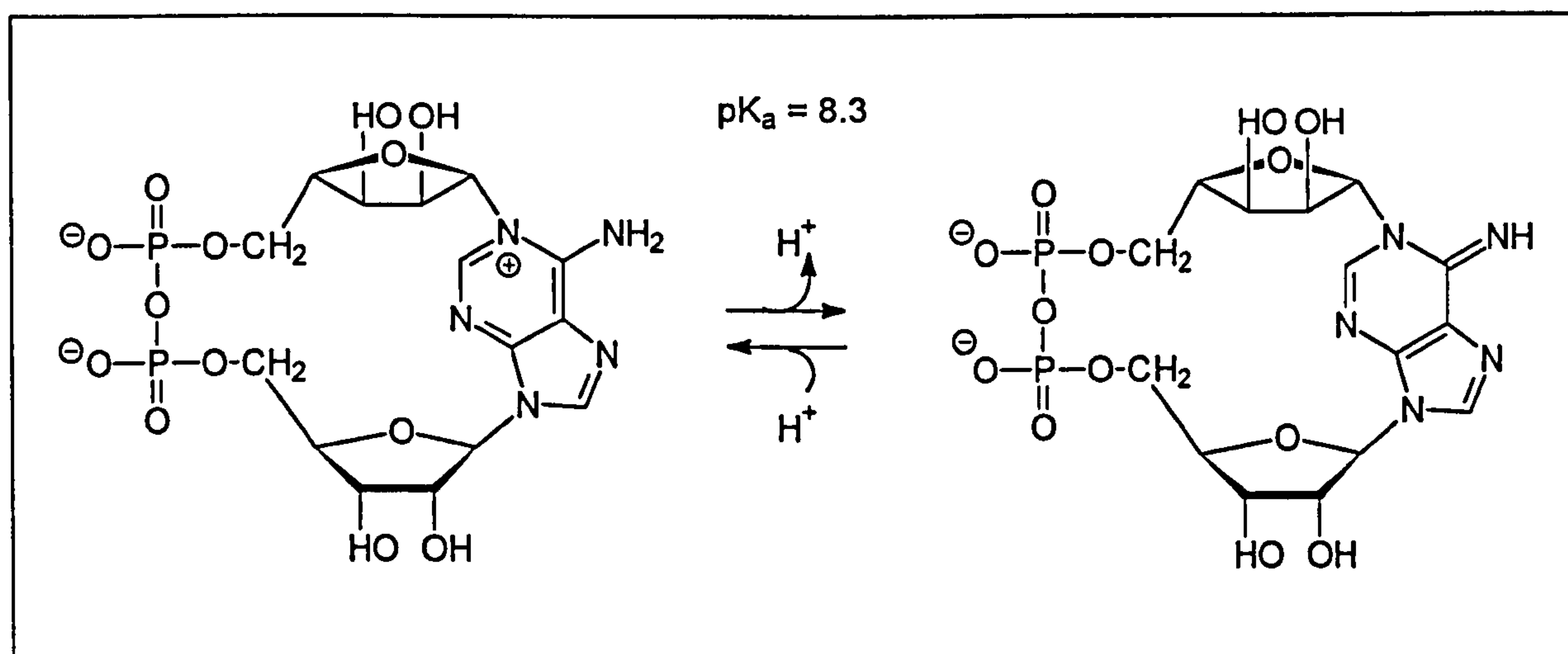
Figure 2.1.1 Acceptor amino acids for mono- and poly(ADP-ribose)ation.²⁹

2.1.2 Cyclic ADP-ribose

Cyclic ADP-ribose (cADPR) is a novel cyclic metabolite of NAD^+ involved in calcium (Ca^{2+}) signalling.^{34,35} The mobilisation of Ca^{2+} from intracellular stores is of critical importance to fundamental processes such as fertilisation, muscle contraction and morphogenesis.³⁸ cADPR also serves as a second messenger for Ca^{2+} for insulin secretion in pancreatic β -cells.³⁹ cADPR is naturally occurring and its endogenous levels in various rat tissues have been determined.⁴⁰ The cyclic nature of this metabolite was recently confirmed by X-ray crystallography⁴¹ which clearly showed the β configuration of the cyclisation linkage. Jacobson *et al*⁴² also demonstrated the β configuration of the

linkage using NMR techniques. From their analyses of the ultraviolet absorption spectra of cADPR as a function of pH, they proposed the following structures (illustrated in *Figure 2.1.2*).⁴²

*Figure 2.1.2 Structures of the protonated and deprotonated forms of cyclic ADP-ribose*⁴²



A pK_a value of 8.3 was determined and it was proposed that conformational changes in the structure of cADPR might be related to its mode of action in the mobilisation of Ca²⁺.⁴²

cADPR is synthesised by ADP-ribosyl cyclase and the hydrolysis of cADPR is catalysed by cyclic ADP-ribose hydrolase. There also exists a class of bifunctional enzymes, which exhibit both of the aforementioned catalytic activities towards cADPR.⁴³ The transmembrane glycoprotein, identified as the human lymphocyte antigen CD38, is an example of such a bifunctional enzyme. CD38 has been shown to catalyse the formation of cADPR from NAD⁺ and its hydrolysis to generate free ADP-ribose.⁴⁴ The rat and murine homologues of CD38 also exhibit bifunctional activities.^{45,46} It has been proposed that an enzyme-stabilised ADP-ribosyl oxocarbenium-ion is involved during the synthesis and hydrolysis of cADPR.^{47,48} This particular feature of NAD⁺ hydrolysis will be discussed in more detail in Section 2.5.

2.2 Poly(ADP-ribose) Polymerase: PARP [EC 2.4.2.30]

Poly(ADP-ribose) polymerase or PARP [also referred to as poly(ADP-ribose) synthetase and poly(ADP-ribosyl) transferase: pADPRT] has been extensively investigated since its discovery in the 1960's.^{30,31} The interest in this highly abundant nuclear enzyme soon flourished as it was quickly realised that the exact biological role of PARP was far from straightforward. Indeed, present day literature continues to indicate that the precise functions of PARP have yet to be fully elucidated.

2.2.1 Poly(ADP-ribosyl)ation

Poly(ADP-ribosyl)ation involves the posttranslational modification of nuclear proteins. Using β -NAD⁺ as a substrate, PARP catalyses the transfer of the ADP-ribose moiety from β -NAD⁺ to a suitable nuclear acceptor protein. The repetition of this catalytic activity results in the formation of negatively-charged homopolymeric chains of repeating ADP-ribose monomers.

Table 2.1 illustrates postranslational modifications of DNA, RNA and chromatin proteins.⁴⁹ Of all the postranslational modifications, poly(ADP-ribosyl)ation may induce the most substantial alteration of chromatin structure.⁵⁰ The modifying residues are variable in both size and structure and polymers of 200 or more ADP-ribose units have been detected.⁵¹ Furthermore, the polymeric chains contain two negative charges per monomer and a highly charged poly-anion in close proximity to chromatin would be expected to have a profound effect on its structure and function.

The alteration of chromatin would inevitably influence the processes of gene expression, DNA replication, repair and recombination,⁵⁰ and consequently PARP has been associated with all of these processes and more (in particular, DNA repair). A number of roles have been proposed for the function of PARP within the cell and the literature concerning these will be discussed in more detail in Chapter Three.

Table 2.1 **Posttranslational modifications of nuclear macromolecules⁴⁹**

Macromolecule	Modification
DNA	Methylation Cutting and splicing (rejoining)
RNA	Internal methylation Polyadenylation 5'-terminal methylation Cutting and splicing (rejoining)
Chromatin Proteins	Phosphorylation Methylation Acetylation ADP-ribosylation

2.3 Structural Conservation of PARP between species.

There is clear evolutionary conservation of PARP among eukaryotic organisms and it has been suggested that this alone provides evidence for a critical role of PARP in biological systems. Purified PARP has been isolated from human placenta,^{52,53} calf thymus,⁵⁴ fish,⁵⁵ and *Helix pomatia*.⁵⁶ PARP enzymic activity has also been detected in plants and lower eukaryotes, such as the slime mould *Dictyostelium discoideum*,⁵⁷ and the diflagellate *Cryptocodinium cohnii*.⁵⁸

Cloning techniques have provided valuable methods which allow the characterisation of the PARP enzyme at the molecular level. PARP cDNAs have been isolated from a number of phylogenetically divergent species, and a comparison of the amino acid sequences revealed a high degree of homology between different classes of animals.⁵⁹ The cDNA for human PARP was cloned by two independent research groups in 1987.^{60,61} Subsequently, cDNAs for mouse,⁶² rat,⁶³ bovine,⁶⁴ chicken,⁶⁵ *Xenopus laevis*,⁶⁶ cherry salmon⁶⁶ and *Drosophila melanogaster*⁶⁷ have been cloned. *Table 2.2* summarises the results obtained from the comparison of PARP cDNAs from different species.⁵⁹

Table 2.2 *Homology in nucleotide and amino acid sequences among PARP cDNAs from different species.*⁵⁹

aa nt	Human	Mouse	Bovine	Chicken	<i>Xenopus</i>	<i>Drosophila</i>
Human		95 (92)	96 (90)	88 (79)	85 (75)	61 (43)
Mouse	87		94 (88)	86 (77)	84 (73)	61 (43)
Bovine	88	85		88 (77)	84 (72)	62 (43)
Chicken	76	74	74		87 (78)	61 (43)
<i>Xenopus</i>	71	70	69	73		61 (43)
<i>Drosophila</i>	50	50	49	51	51	

aa = amino acid sequence homology, shown as % similarity (% identity in parentheses)
 nt = nucleotide sequence homology

The amino acid sequences show a very high percentage of homology among mammals. *Drosophila* PARP, on the other hand, has only 61% similarity (43% identity) in amino acid sequence to human PARP, which could be attributable to the evolutionary distance between the two species.⁵⁹ However, the possibility that *Drosophila* PARP has a function specific to the invertebrate enzyme should not be ruled out.⁶⁸ When only the vertebrate sequences are considered, the overall conservation is 62%, which is reduced to only 32% when the *Drosophila* PARP sequence is included.⁶⁹ The strictly conserved regions of the amino acid sequences are largely associated with peptide sequences which are required for enzymatic activity.

Recently, the cloning and characterisation of the first plant cDNA encoding a PARP homologue was reported.⁷⁰ Evidence for poly(ADP-ribosyl)ation in plants was first obtained from investigations on germinating seeds⁷¹ and cytological work with onion tissues.⁷² The plant PARP homologue shows approximately 62-63% similarity (41-45% identity) to the catalytic domains of mammals, *Xenopus* and insect cDNAs. However, there is little similarity between the *N*-terminal sequences.⁷⁰ In line with animal PARP, plant PARP has also been suggested to be involved in DNA repair.

In the following section, important structural features of the PARP enzyme (excluding the plant homologue) will be discussed.

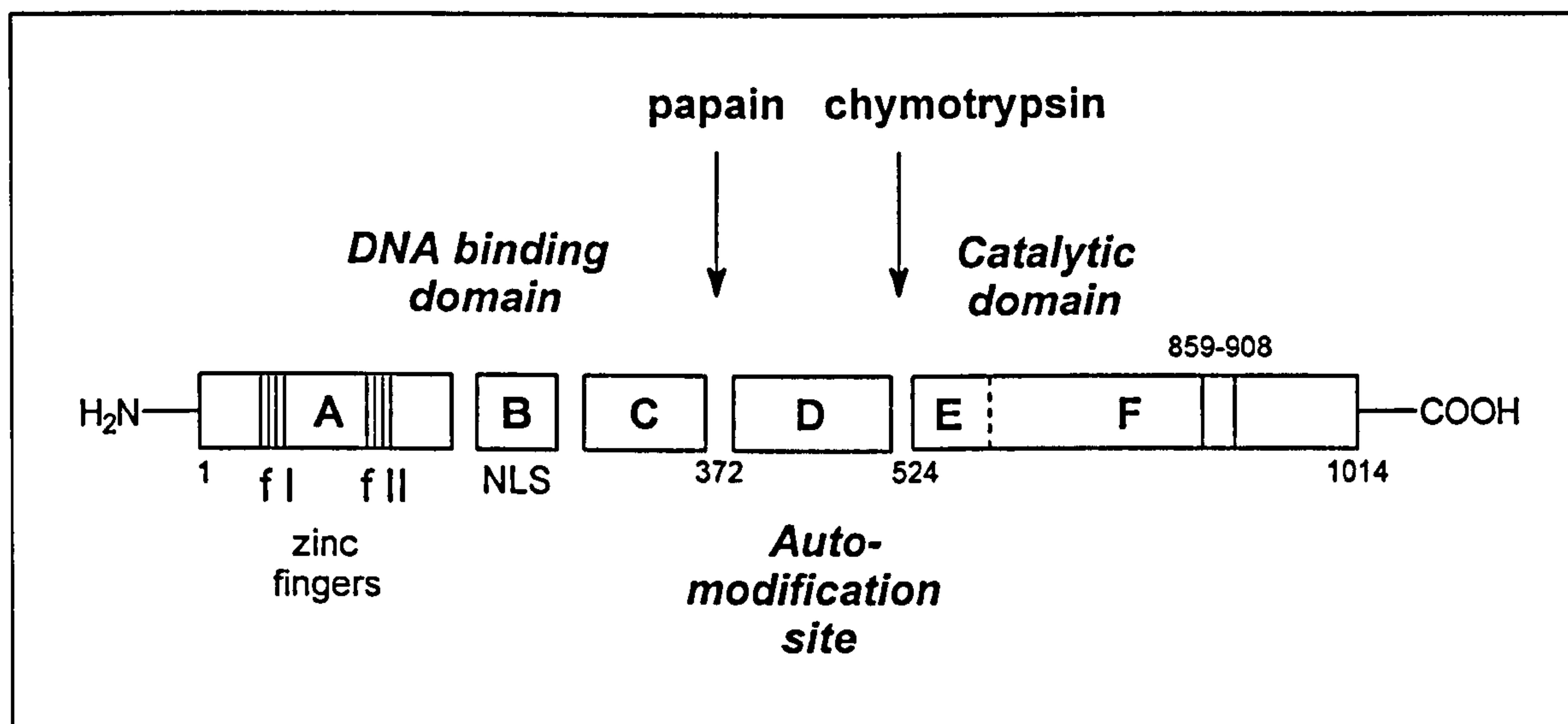
2.4 Modular Organisation of PARP

PARP is composed of 1014 amino acids and has a molecular weight of 116 kDa.⁷³ The enzyme's activity is strictly dependent on the presence of DNA strand breaks⁷⁴ and PARP has been implicated as a critical regulatory component of the cellular response to DNA damage. Zahradka and Ebisuzaki⁷⁵ demonstrated that PARP is a metalloenzyme and requires zinc for its activity. Crucial information about the enzyme structure was revealed when Kameshita *et al* showed that partial proteolysis with papain and/or α -chymotrypsin yielded three major proteolytic fragments:^{76,77}

- (i) a 46 kDa amino-terminal fragment, identified as the DNA-binding domain (DBD),
- (ii) a central 22 kDa fragment containing sites for poly(ADP-ribosyl)ation, known as the automodification domain,
- (iii) a 54 kDa carboxyl-terminal fragment possessing the site for accepting NAD⁺ (the substrate-binding domain).

Figure 2.4 shows a schematic representation of the functional domains of PARP

Figure 2.4 Schematic representation of the three functional domains of human PARP^{68,77}



NLS = nuclear localisation signal

The arrows indicate the cleavage sites for papain and α -chymotrypsin and, interestingly, these sites correspond to the two regions of lowest conservation.^{68,69}

2.4.1 The DNA-Binding Domain (DBD)

The DBD consists of three subdomains A, B and C (*Figure 2.4*).

The Zinc Fingers

PARP is a zinc metalloenzyme which has been shown to contain two zinc ions per PARP molecule.⁷⁸ These zinc ions are coordinated to cysteine and histidine ligands (a total of four ligands per zinc ion), resulting in secondary protein structures known as zinc fingers. The two zinc fingers of PARP, f I and f II, are located in residues 21-56 and 125-162, respectively (domain A, 29 kDa, *Figure 2.4*).

Experiments involving metal depletion and restoration revealed that Zn(II) was crucial for the DNA-binding capability of domain A.⁷⁸ Since the absence of zinc results in a

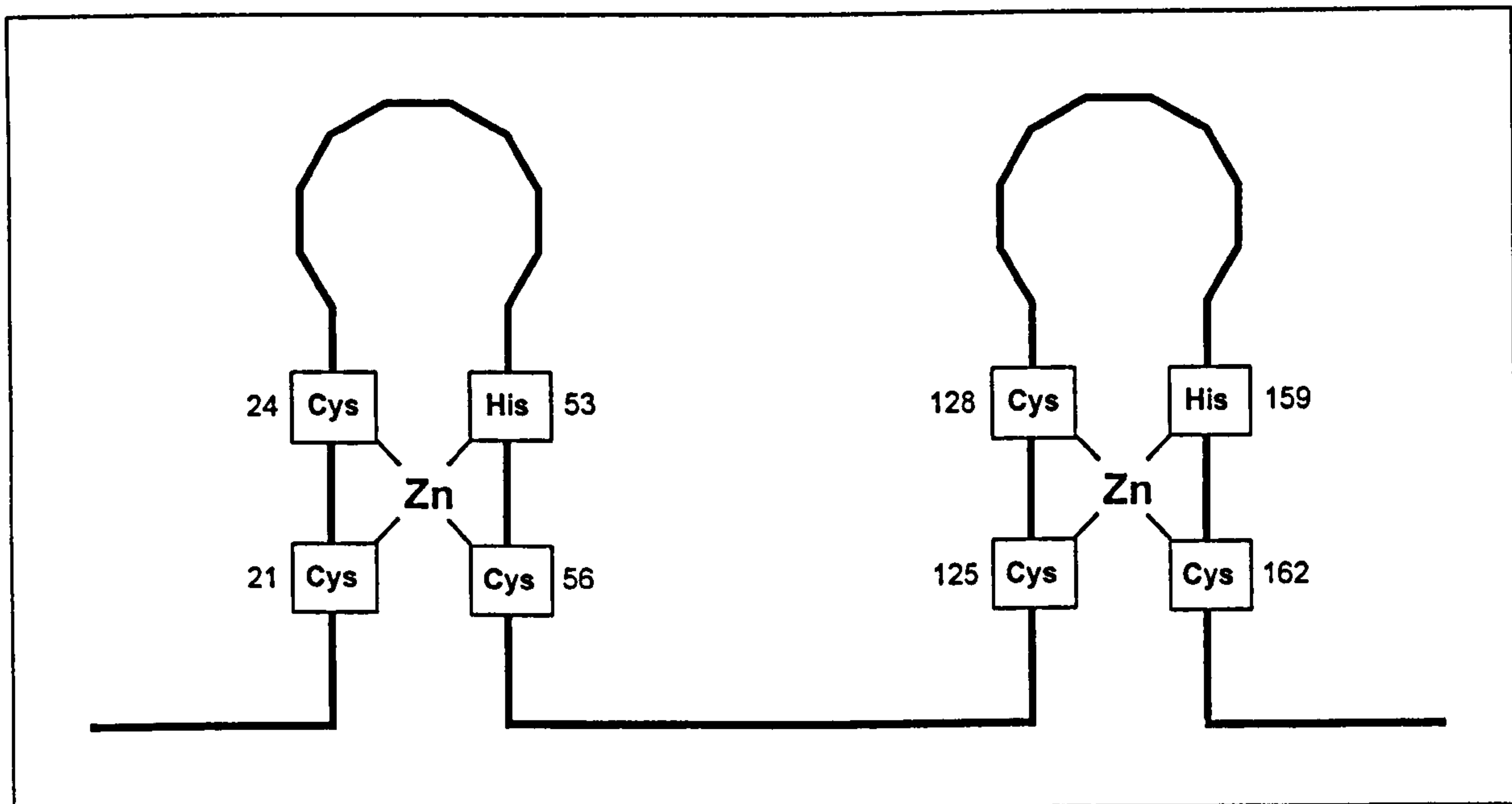
decrease in enzymatic activity, it has been hypothesised that reduced PARP activity may be associated with a nutritional zinc deficiency.⁷⁹ A nutritional zinc deficiency can result in abnormalities of gene expression, cell division and growth, and since PARP has been associated with these biological processes, it has been suggested that the decrease of PARP activity could be, at least partially, responsible for the effects seen.⁷⁹

The zinc fingers of PARP are rather unusual in their lack of sequence homology to other existing zinc-finger classes.⁸⁰ They also have longer loops, of 28-30 amino-acid residues, than those of most other DNA-binding proteins.⁸¹

Ikejima *et al* have demonstrated that each zinc finger has a specific function.⁸² It appears that on disruption of f I, sensitivity to double-stranded DNA breaks is abolished, whereas the enzymatic activity which depends on single strand DNA breaks no longer remains when f II is disrupted. In other words, the zinc-fingers f I and f II recognise double- and single-stranded DNA breaks, respectively.⁸² Gradwohl *et al* confirmed these findings by generating a library of PARP-DBD mutants possessing mutations in either one or both of the zinc fingers, and testing them for their ability to bind DNA breaks.⁸³ However, regardless of the nature of the activating DNA break, the first zinc finger (f I) is absolutely required for PARP catalytic activity.⁸²

Since the two zinc fingers are essential for the recognition and binding of damaged DNA, it would be reasonable to suggest that these structures may be conserved among the eukaryotes. Mazen *et al* performed experiments in which critical residues, involved in metal coordination of the two zinc fingers, were identified.⁸⁴ Comparison of the cDNA sequences of human, mouse, bovine, chicken, *Xenopus* and *Drosophila* PARP revealed that all of the ligands of the first and second zinc fingers (Cys21, Cys24, His53, Cys56 and Cys125, Cys128, His159, Cys162, respectively) are strictly conserved. The zinc finger motifs are of the form $CX_2CX_{28-30}HX_2C$, where C = cysteine, H = histidine and X = any amino acid.⁸² *Figure 2.4.1* shows a schematic representation of the zinc finger motifs and the conserved chelating amino acid residues.

Figure 2.4.1 Schematic representation of the zinc fingers present in the DBD of PARP



There are also some basic residues in the DBD that are strictly conserved: Arg18, Lys30, Arg34, Arg122, Lys134 and Arg138. It has been proposed that these basic residues may be significant with respect to their interaction with DNA.⁵⁹

The Nuclear Localisation Signal (NLS)

Domain B of the DBD contains a nuclear localisation signal (NLS), which consists of a short amino acid sequence (residues 207-226) generally rich in basic residues. This sequence serves to target proteins larger than 40-60 kDa into the cell nucleus.⁶⁸ Schreiber *et al* found that the human NLS is composed of two basic clusters, separated by eleven amino acids, and belongs to the class of bipartite signals.⁸⁵ Site-directed mutagenesis located the amino acid residue Lys222, in the second basic cluster and this lysine residue was identified as critical for nuclear-targeting activity.⁸⁵

On proteolytic degradation of PARP with plasmin,⁸⁶ the cleavage site occurs immediately after the crucial residue Lys222, suggesting that this cluster of residues is situated on the surface of the PARP molecule.

Cleavage of the NLS of PARP is also of significance in the process of apoptosis (or programmed cell death). The involvement of PARP in this process will be discussed in Section 3.2.7.

Domain C

At present, no known function with relation to enzymatic activity has been ascribed to domain C of the DBD. However, Trucco *et al* used random mutagenesis techniques to identify two critical residues in domain C and suggested the possibility that this domain may contribute, in some way, to stimulation of catalytic activity by DNA strand breaks.⁸⁷

2.4.1.1 Activation of PARP by DNA strand breaks

Early investigations on the activation of PARP by DNA molecules containing different types of strand breaks were carried out by Benjamin and Gill.⁷⁴ They constructed a range of defined DNA fragments of known structure and determined the stimulatory effects on polymer synthesis by PARP. Their results indicated that single-stranded intact DNA was ineffective in the activation of PARP whereas double-stranded breaks with blunt ends produced the highest stimulation of activity.⁷⁴

In contrast, recent reports have indicated that PARP preferentially binds to single-strand breaks in double-stranded DNA.^{88,89} Ménissier de Murcia *et al* used a 'footprint' assay to investigate the interaction between PARP and nicked DNA. They found that PARP symmetrically 'protects' 7 (\pm 1) nucleotides of the continuous DNA strand on either side of the nick and that the protection is independent of DNA sequence.⁸⁸ It is interesting to note that, under the experimental conditions used, no footprint was observed between PARP and methylated DNA or PARP and blunt-ended double-stranded DNA. The authors suggested that this may be due to the enzyme recognising the continuity of the DNA ribose-phosphate backbone.⁸⁸

Further information on the binding of PARP to single-stranded DNA lesions was presented by Le Cam *et al*, who used electron microscopy (EM) techniques to visualise a

139 base-pair DNA fragment containing a central single-strand break.⁸⁹ EM showed the nicked duplex to have a V-like structure compared to the intact control duplex. The result of incubation of PARP with the nicked DNA construct, was the binding of the enzyme to the apex of the V-shape, causing an increase in angle values of the structure.⁸⁹

2.4.2 The Automodification Domain

The automodification domain (domain D, *Figure 2.4*) contains the acceptor sites for poly(ADP-ribose). Poly(ADP-ribosyl)ation involves the transfer of poly(ADP-ribose) to nuclear acceptor proteins including PARP itself. The synthesis of polymers on the PARP enzyme is referred to as automodification whereas poly(ADP-ribosyl)ation of other nuclear proteins is referred to as heteromodification. The PARP enzyme has been found to be the major acceptor protein of poly(ADP-ribose) both *in vitro*^{90,91} and in DNA-damaged cells.⁹²

It has previously been reported that the number of acceptor sites present in the automodification domain is fifteen. These sites are all highly conserved glutamic acid residues.⁹¹ However, in a subsequent report by Desmarais *et al*, it was found that these acceptor sites were not the only sites present in the PARP molecule.⁹³ The net result of automodification of PARP is its catalytic inactivation by dissociation from the DNA strand it is bound to. This presumably occurs through electrostatic repulsion between the negatively charged enzyme-bound polymers and DNA itself. Desmarais *et al* found a total of 13 acceptor sites outside the automodification domain and suggested that modification by PARP at these sites may provide a more efficient way for the enzyme to modulate its repulsion from DNA.⁹³

The Leucine-Zipper Motif

The presence of a leucine-zipper motif in the automodification domain of PARP was first demonstrated in *Drosophila melanogaster* by Uchida and coworkers.⁶⁷ Leucine-zipper proteins, with periodic repetitions of leucine residues, have been proposed to mediate the formation of homo- and heterodimers. This dimerisation may be involved in the DNA-

binding and functions of the leucine-zipper proteins.⁶⁷ Uchida *et al* proposed that the leucine-zipper motif of PARP may serve to allow protein-protein interactions between PARP and nuclear acceptor proteins.⁶⁷

Despite the automodification domain being less conserved than the DBD and the catalytic domain, comparison of the cDNA sequences of human, mouse, bovine, chicken, *Xenopus* and *Drosophila* revealed that within the leucine-zipper motif, two glutamic acid residues (Glu401 and Glu407) are strictly conserved in all six species.⁵⁹ These glutamate residues may be acceptor sites for automodification.⁹¹ If these sites were poly(ADP-ribosyl)ated, dissociation of the PARP dimer may occur⁶⁷ and automodification of these residues may, thus, be a means of regulating dimerisation.

2.4.3 The Catalytic Domain

The catalytic domain of PARP (domains E and F, 54 kDa, *Figure 2.4*) contains the NAD⁺-binding region and is responsible for the catalytic activity of the enzyme. NAD⁺ commonly serves as a coenzyme in electron-transfer reactions and its utilisation as a substrate by PARP is rather unusual.

The carboxyl-terminal region of PARP shows the highest sequence homology between species. The sequence from residues 859-908 (domain F, *Figure 2.4*) is known as the *PARP signature* and is strictly conserved (100 %) among the vertebrates (92 % among all species).⁶⁸

Two major NAD⁺-binding residues, Trp1014 (the C-terminal residue) and Lys893 (located within the PARP signature), were identified by photolabelling experiments carried out by Kim *et al*.⁹⁴ The NAD⁺ photoaffinity analogue 2-azido-[α -³²P]NAD⁺ was used to label residues in the catalytic domain of human PARP. Their results suggested that Lys893, but not Trp1014, was crucial for enzymatic activity, although the deletion of Trp1014 along with residues 1007-1013, resulted in the abolition of catalytic activity, indicating that Trp1014 is in some way necessary.⁹⁴ It was also shown, from saturation

experiments which determined stoichiometry, that PARP contains a single NAD⁺-binding site.⁹⁴

Simonin *et al* demonstrated that the PARP catalytic domain can be shortened to a 40 kDa unit (domain F, *Figure 2.4*) without losing basal polymer synthesis activity.⁹⁵ Furthermore, this basal activity is independent of the presence of DNA.⁹⁵ When the cDNA encoding domain F was overproduced and purified in *E. Coli*, it was found that the affinity for NAD⁺ associated with the 40 kDa fragment was comparable to that of full-length PARP. However, the 40 kDa unit had a 500-fold lower specific activity when compared to full-length PARP activated by DNA strand breaks.⁹⁶

The possibility that the DBD of PARP is capable of transducing some sort of signal to the catalytic domain, once bound to a DNA strand break, was investigated by Trucco *et al*.⁸⁷ They identified the residue Lys97, located in domain A between the two zinc fingers, and suggested that this residue could be involved in the relay of information between the DBD and the catalytic domain.⁸⁷

2.5 Catalytic Activities of PARP

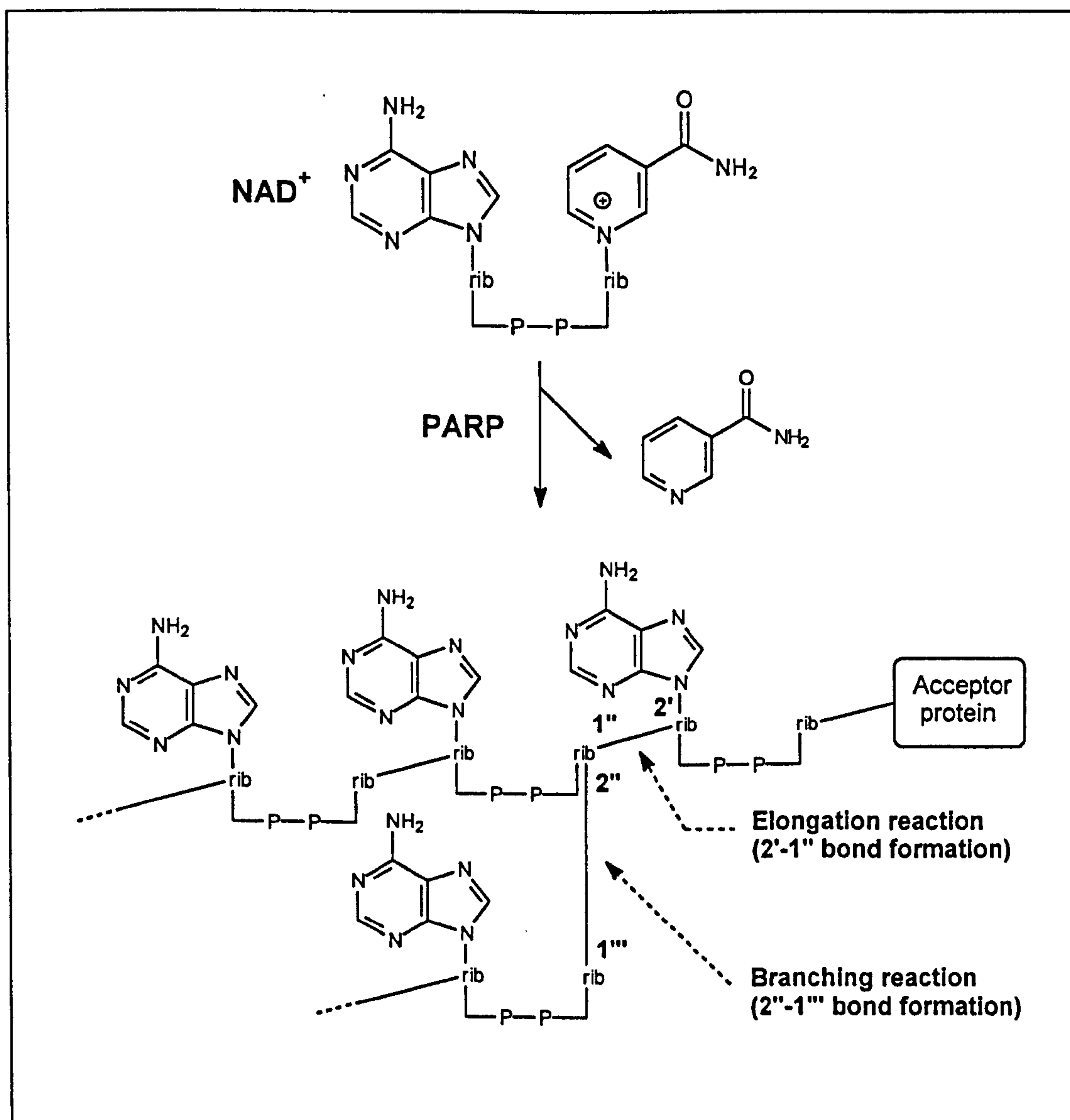
2.5.1 Automodification

During poly(ADP-ribose) synthesis, PARP is responsible for the catalysis of three chemically distinct enzymatic steps:⁹⁷

- (i) the glutamate-specific attachment of an ADP-ribose moiety to an acceptor protein (initiation reaction),⁹⁸
- (ii) the formation of a (2'-1'') ribose-ribose glycosidic bond (polymer elongation reaction),⁹⁹
- (iii) the formation of (2''-1''') ribose-ribose bonds (branching reaction).¹⁰⁰

Figure 2.5.1.1 shows a schematic representation of the structure of NAD^+ . The initiation of polymer synthesis results in the attachment of an ADP-ribose unit to an acceptor protein with the elimination of nicotinamide. The broken arrows indicate sites of elongation and branching.

Figure 2.5.1.1 Schematic representation of the catalytic activities of PARP²⁹

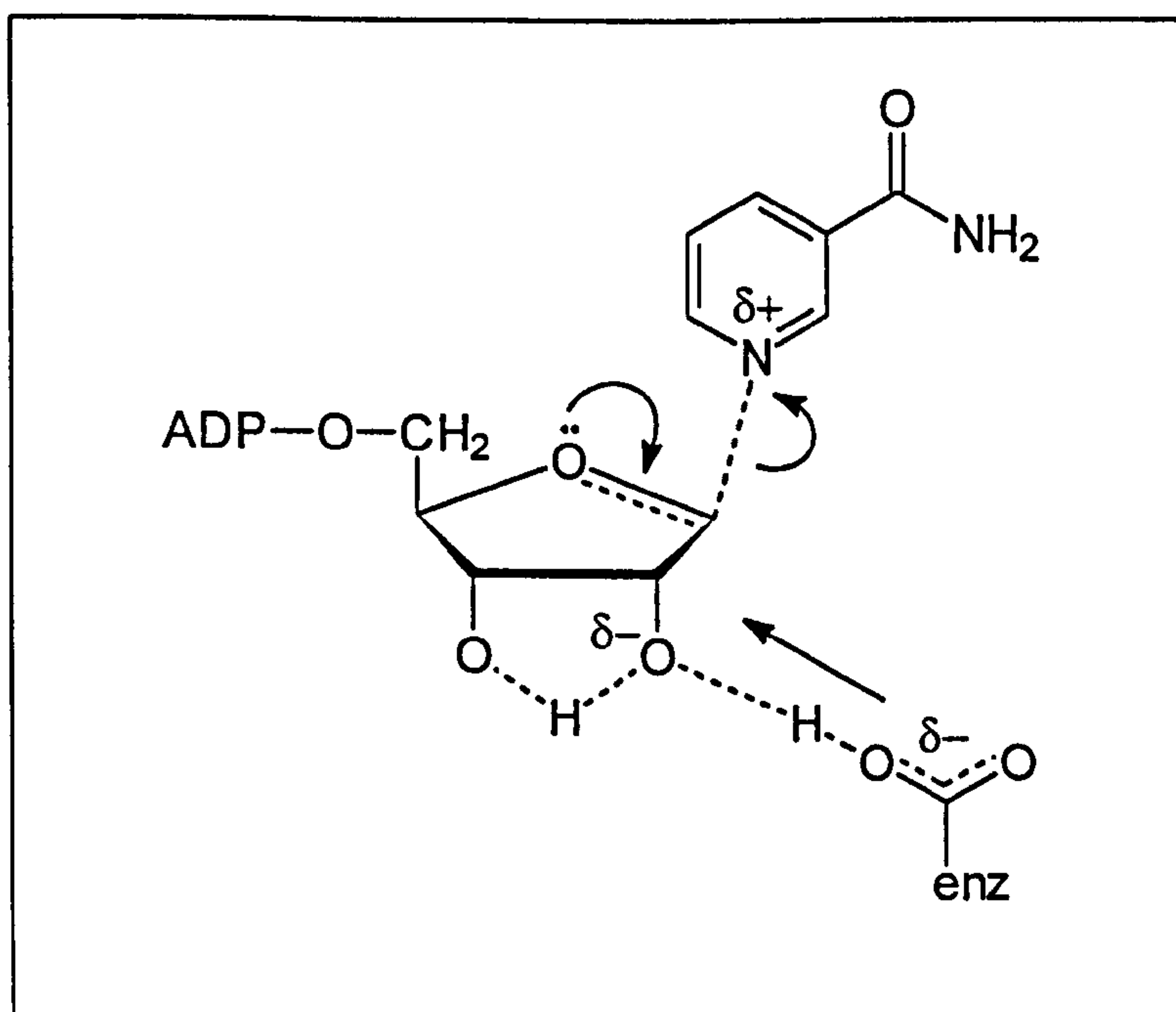


rib = ribose P = phosphate

Studies to establish the mechanism of cleavage of the ribose-nicotinamide bond indicate the involvement of an enzyme-stabilised oxocarbenium-ion intermediate.¹⁰¹⁻¹⁰³ This stabilisation can only occur if the ribose group adopts a conformation within the enzyme active-site which results in maximum overlap of the lone-pair electrons on the oxygen with the adjacent vacant *p* orbital. Thus, loss of nicotinamide would be assisted by the ribose oxygen lone-pair.

A recent model for the stabilisation of the oxocarbenium-ion intermediate was proposed by Oppenheimer (see *Figure 2.5.1.2*).¹⁰³ This model predicts the presence of an anionic basic residue, possibly a glutamate, in the active-site. However, the precise mechanistic details of the enzymatic cleavage of the ribose-nicotinamide bond are not fully understood.

Figure 2.5.1.2 *Proposed mechanism for the inductive stabilisation of an oxocarbenium-ion intermediate by an active-site carboxylate.*¹⁰³



In order to investigate the molecular mechanism of the automodification reaction (*i.e.* where PARP acts as the acceptor for the polymer), Alvarez-Gonzalez and Mendoza-Alvarez performed kinetic experiments in which they measured the initial rates of automodification at physiological NAD^+ concentration.¹⁰⁴ Since the initial rates of automodification, as a function of PARP concentration, increased with second order kinetics, they concluded that two molecules of PARP (*i.e.* a catalytic dimer) are necessary for auto-mono(ADP-ribosyl)ation (the initiation reaction) and auto-poly(ADP-ribosyl)ation (elongation and branching).¹⁰⁴ It was also found that the initial rates of automodification increased with second order kinetics as a function of substrate concentration, indicating that the catalytic PARP dimer requires two molecules of NAD^+ .¹⁰⁴

The suggestion of a catalytic dimer is in agreement with the findings of Bauer and coworkers, who observed the macromolecular association of PARP into oligomeric forms.¹⁰⁵ Furthermore, Panzeter and Althaus demonstrated that optimal polymerisation activity of PARP was obtained at a stoichiometry of two enzyme molecules per molecule of DNA.¹⁰⁶ However, the individual molecular functions of each monomer in the catalytic dimer are presently unclear. It is not known whether one monomer acts as catalyst whilst the other acts as acceptor or whether both function simultaneously, as catalyst and acceptor.¹⁰⁴

The automodification reaction of PARP has been dissected into the individual reactions of initiation, elongation and branching, by manipulating the availability of $\beta\text{-NAD}^+$, the ADP-ribosylation substrate.¹⁰⁷

The Initiation Reaction

The polymer initiation reaction has been shown to proceed *via* a distributive mechanism.¹⁰⁴

Simonin *et al* observed that the carboxyl-terminal domain of PARP shares sequence similarities with the NAD(P)^+ -binding domains of the leucine and glutamate

dehydrogenases (L/G DH).⁹⁶ Using site-directed mutagenesis, they identified critical residues in the active site of human PARP, Lys893 and Asp993. By superimposing the C-terminal domain sequence of PARP on the recently solved three-dimensional structure of the NAD⁺-dependent glutamate dehydrogenase (GDH) from *Clostridium symbiosum*,¹⁰⁸ they found that the residues they had identified were conserved between the two enzymes and were located near or in the catalytic cleft.⁹⁶

According to Baker *et al*, there is a cluster of basic residues present in the GDH structure which has been proposed to be involved in stabilising glutamate binding.¹⁰⁸ An analogous cluster of residues is present in the PARP enzyme, one of which is Lys893, identified as being essential for activity.^{94,96} Since modification by PARP preferentially occurs at a glutamate residue within a polypeptide chain, Simonin *et al* postulated that the cluster of basic residues present in PARP may be involved in stabilisation of the negative charge on the acceptor amino acid, allowing its correct positioning with respect to the NAD⁺ molecule.⁹⁶

Asp993, the other important residue identified, is located in a particular type of structure known as the *Rossmann Fold*.¹⁰⁹ The Rossmann Fold is a characteristic NAD⁺-binding structure for many of the NAD⁺-dependent dehydrogenases and a similar structure has been found in the catalytic domain of PARP. Asp993 has been implicated in the binding of the ADP-ribose group of the NAD⁺ molecule, again according to the GDH model.⁹⁶

However, a second model for the involvement of residues in the initiation reaction was proposed by Marsischky *et al*, who attributed their findings to the sequence similarities between the catalytic domain of PARP and the analogous region of the mono(ADP-ribosyl)ating bacterial toxins.¹¹⁰ It is proposed that many of the mono(ADP-ribosyl)ating toxins share a similar type of reaction mechanism involving a particular glutamic acid residue.¹¹¹ This residue is thought to be involved in the catalysis reaction of the mono(ADP-ribosyl) transferases and, since Glu988 of PARP is in the analogous position it was suggested to play a similar role.¹¹⁰

In contrast, the cDNA isolated from the slime mould *Dictyostelium discoideum* was found to contain a glutamine residue in position 988.¹¹² The capacity of *Dictyostelium* PARP to synthesise ADP-ribose polymers was clearly demonstrated, with the detection of polymers of up to 100 ADP-ribose units in length.¹¹² Its enzymic activity was however, found to be markedly reduced compared to the human enzyme.

The Elongation Reaction

The elongation reaction catalysed by PARP has been shown by a number of groups to proceed *via* a protein-distal elongation mechanism.¹¹³⁻¹¹⁵ Distal addition involves the 2'-hydroxyl group of the growing polymer (polymer tail) acting as the acceptor for the covalent addition of the next ADP-ribose moiety (illustrated in *Figure 2.5.1.3*). Each addition of monomer results in the elimination of a molecule of nicotinamide.

For every initiation reaction, it has been estimated that PARP has the capacity to catalyse 5-7 branching reactions and more than 200 elongation reactions, indicating that the elongation of the polymer appears to be the main catalytic function.¹¹⁶

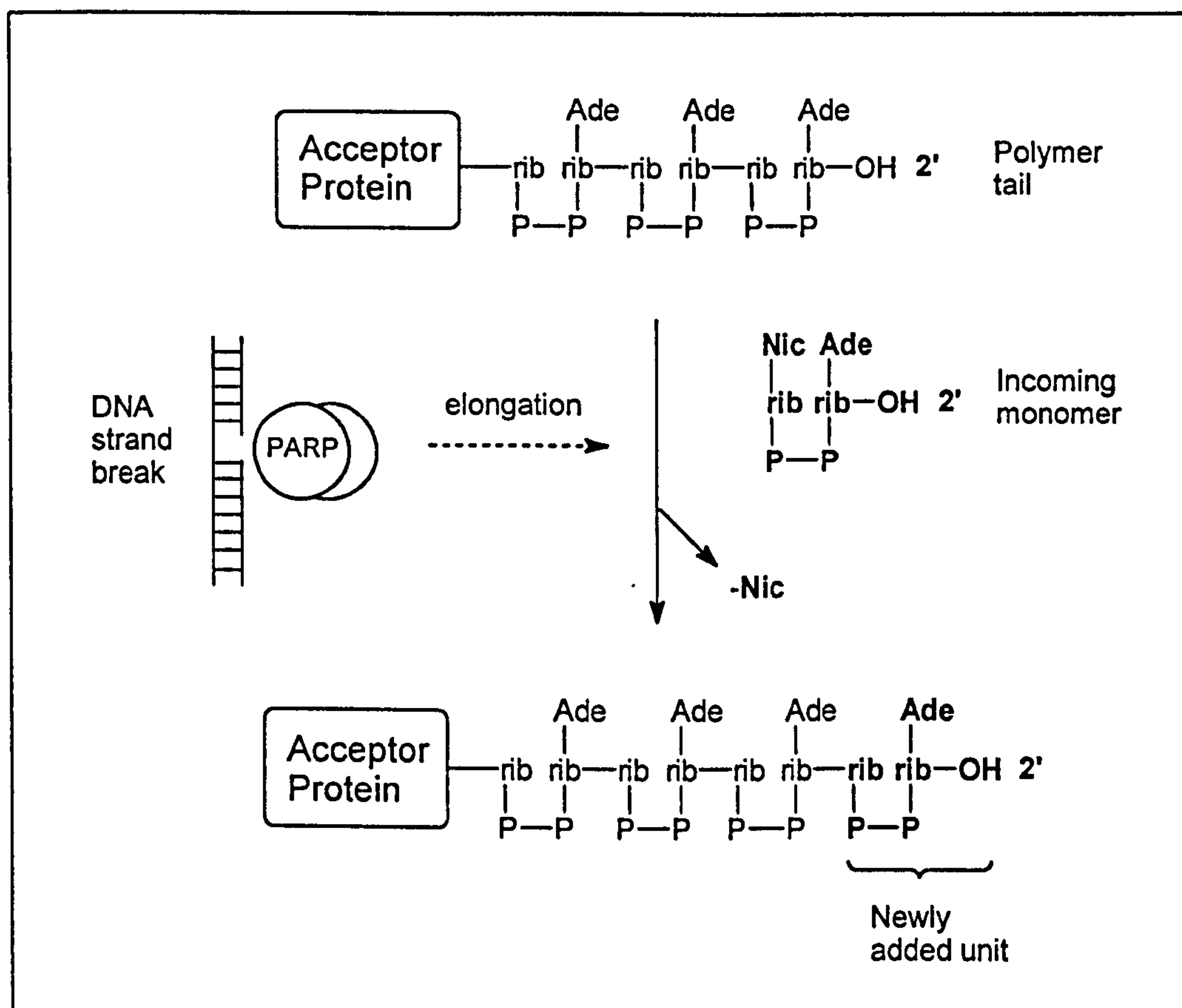
The Branching Reaction

Highly complex, branched ADP-ribose polymers have been detected containing over 200 ADP-ribose units and 5-6 branching points per molecule.¹¹⁶ These branched structures have also been observed using electron microscopy.¹¹⁷

2.5.2 Heteromodification

Heteromodification involves the poly(ADP-ribosyl)ation of histones and DNA metabolising enzymes. Details of the molecular mechanism of the heteromodification reaction have not yet been elucidated. It is likely that heteromodification will also be bimolecular in nature, and it is thought that the heterodimerisation of PARP with poly(ADP-ribose) acceptor proteins involves leucine zipper motifs.⁹⁸

Figure 2.5.1.3 The protein-distal addition mechanism for poly(ADP-ribose) elongation



Ade = adenosine Nic = nicotinamide
 rib = ribose P = phosphate

2.6 Catabolism of Poly(ADP-ribose)

A balance must exist between the anabolism and catabolism of the polymer within the cell. The accumulation of long negatively-charged polymers in the near vicinity of chromatin and its associated proteins could only be expected to be detrimental in the long term.

To recap, PARP is activated by the presence of DNA strand breaks. The enzyme recognises and binds to the damaged site *via* the two zinc fingers present in the DBD, and proceeds to synthesise ADP-ribose polymers, using β -NAD⁺ as a substrate. These

polymers are either covalently attached to PARP itself, or to other suitable nuclear acceptor proteins. The automodification of PARP results in its dissociation from the site of damage *via* electrostatic repulsion from DNA, and renders the enzyme covalently modified and enzymatically inactive.

An equally important factor in polymer metabolism is catabolism (degradation of the polymer). The main enzymes involved in poly(ADP-ribose) degradation are poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl protein lyase.

2.6.1 Poly(ADP-ribose) Glycohydrolase (PARG)

The discovery of PARG was first reported in the early 1970's.¹¹⁸⁻¹²⁰ PARG is the principle enzyme responsible for the degradation of poly(ADP-ribose). Due to its synergistic role with PARP, PARG is present in all eukaryotic cells, except yeast.¹²¹ Four nuclear isoforms of the purified enzyme have been detected¹²² and the existence of a cytoplasmic PARG has been reported¹²³ although this is in contrast to reports of exclusively nuclear PARG.^{124,125}

PARG is responsible for the reversal of the automodified state of PARP, and assists in the restoration of the DNA-binding ability and catalytic activity of the enzyme. PARG degrades both linear and branched polymers by hydrolysing the *O*-glycosidic bonds between each monomer.¹²¹ This results in the generation of free ADP-ribose units. This degradative reaction is responsible for the short half-life of poly(ADP-ribose) which, at high levels of DNA damage, may be less than 40 seconds. In contrast, a half-life of 7.7 hours has been observed for constitutive polymer fractions in undamaged cells.¹²⁶

It has been proposed that the mode of action of PARG is a combination of endo- and exoglycosidic action. This was initially suggested by Ikejima and Gill¹²⁷ and later demonstrated by Brochu *et al.*¹²⁸ It appears that polymer catabolism is a highly organised process, where large polymers (greater than 20 residues) are degraded in a fast and processive reaction by endoglycosidic action, and shorter polymers are degraded in a

slower, distributive reaction mode with exoglycosidic action.^{129,130} PARG also hydrolyses branch points of poly(ADP-ribose), although relatively slowly compared to linear polymers.¹³⁰

Until recently, PARG was thought to degrade polymers down to the protein-proximal ADP-ribose residue, which is then removed by ADP-ribosyl protein lyase. Desnoyers *et al* found that PARG was also able to remove the last residue on PARP, indicating the flexibility of the glycohydrolase enzyme.¹²⁹

2.6.2 ADP-ribosyl Protein Lyase

ADP-ribosyl protein lyase was first discovered by Okayama and coworkers¹³¹ and is responsible for the hydrolysis of the bond between the protein-proximal ADP-ribose residue and the glutamate residue on the acceptor protein.¹³² In light of the findings of Desnoyers *et al*, ADP-ribosyl protein lyase may share this activity with PARG.¹²⁹

2.7 Distribution of Poly(ADP-ribose) Metabolising Enzymes Within the Cell

There have been several estimates of the number of PARP molecules present, which ranges from 2×10^5 to 2×10^6 molecules per cell in various cell lines.^{133,134} PARG is, in contrast, approximately 13- to 50-fold less abundant,^{124,135} although its specific catalytic activity is higher.¹³⁴

More than 95% of PARP molecules are localised in the nucleus, although the distribution is uneven as the bulk of the activity is associated with chromatin.¹³⁶ The possibility of different functional classes of PARP molecules existing within the nucleus was raised by Kaufmann *et al*.¹³⁷ However, knowledge of these putative multiple classes is minimal at present.

The detection of cytoplasmic PARP has been reported in free ribosomes and polysomes of HeLa cells¹³⁸ and in mouse messenger ribonucleoprotein (mRNP) particles.¹³⁹ The cytoplasmic PARP isolated from mouse mRNP has an apparent molecular weight of 116 kDa, as does nuclear PARP. However, the enzymatic activity of cytoplasmic PARP is unaffected by the presence/absence of DNA.¹³⁹ As mentioned in Section 2.4.1 nuclear PARP contains a nuclear localisation signal (NLS) which retains the enzyme in the nucleus. The presence of a non-functional NLS has no effect on catalytic activity.⁸⁵ It has been postulated that a posttranslational modification of the NLS (*e.g.* phosphorylation by a protein kinase), could be responsible for the cytoplasmic localisation of the mRNP PARP.¹³⁹

It is also interesting to note that Jesser *et al* detected the copurification of RNA in the cytoplasmic PARP preparation from mRNP.¹³⁹ Desnoyers *et al* also suggested a possible interaction between PARP and RNA.¹⁴⁰ The implications of these observations (*e.g.* the possibility that PARP can bind to RNA *in vivo*), require further investigation.

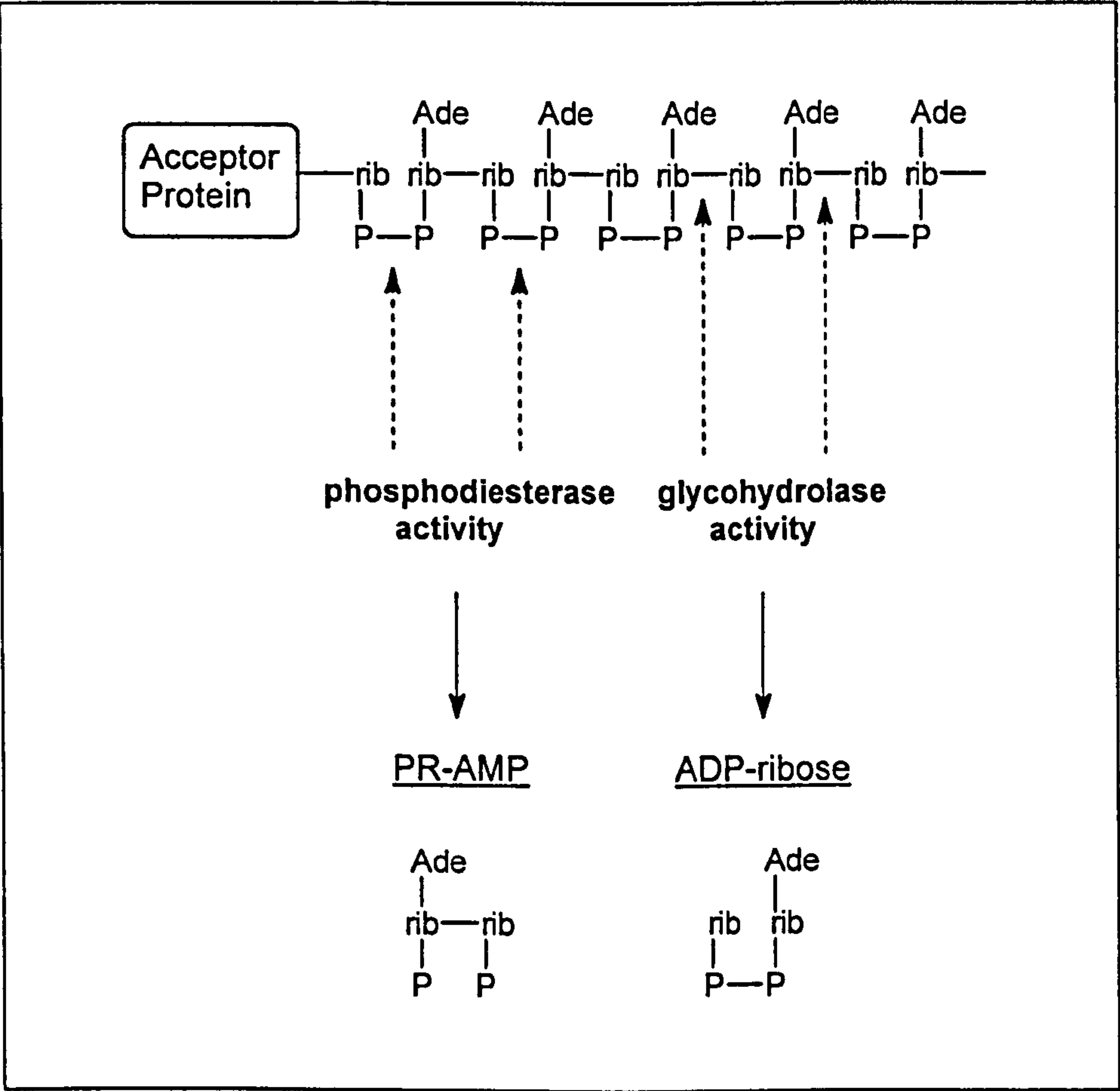
2.7.1 Detection of Poly(ADP-ribose)

Poly(ADP-ribose) is usually characterised by the chromatographic identification of its enzymatic hydrolysis products. As discussed in Section 2.6, catabolism of poly(ADP-ribose) is mainly carried out by the glycohydrolase PARG. However, the polymer can also be hydrolysed by a second class of enzymes, namely the phosphodiesterases.

The analytical enzyme primarily used for the characterisation of poly(ADP-ribose) is snake venom phosphodiesterase [EC 3.1.4.1] (also known as nucleotide phosphodiesterase).⁴⁹ This enzyme hydrolyses the pyrophosphate bond of poly(ADP-ribose) to give one molecule of phosphoribosyl adenosine monophosphate [PR-AMP: 2' (or 3')-(5''-phosphoribosyl)-5'-AMP] from every internal residue, and one molecule of 5'-adenosine monophosphate (5'-AMP) from every non-reducing terminus.^{49,81} This results in a ribose-phosphate group covalently attached to the acceptor protein.

Thus, the major product from enzymatic hydrolysis by PARG is ADP-ribose, whereas the major product of phosphodiesterase hydrolysis is PR-AMP. This is illustrated in *Figure 2.7.1.1*.⁴⁹

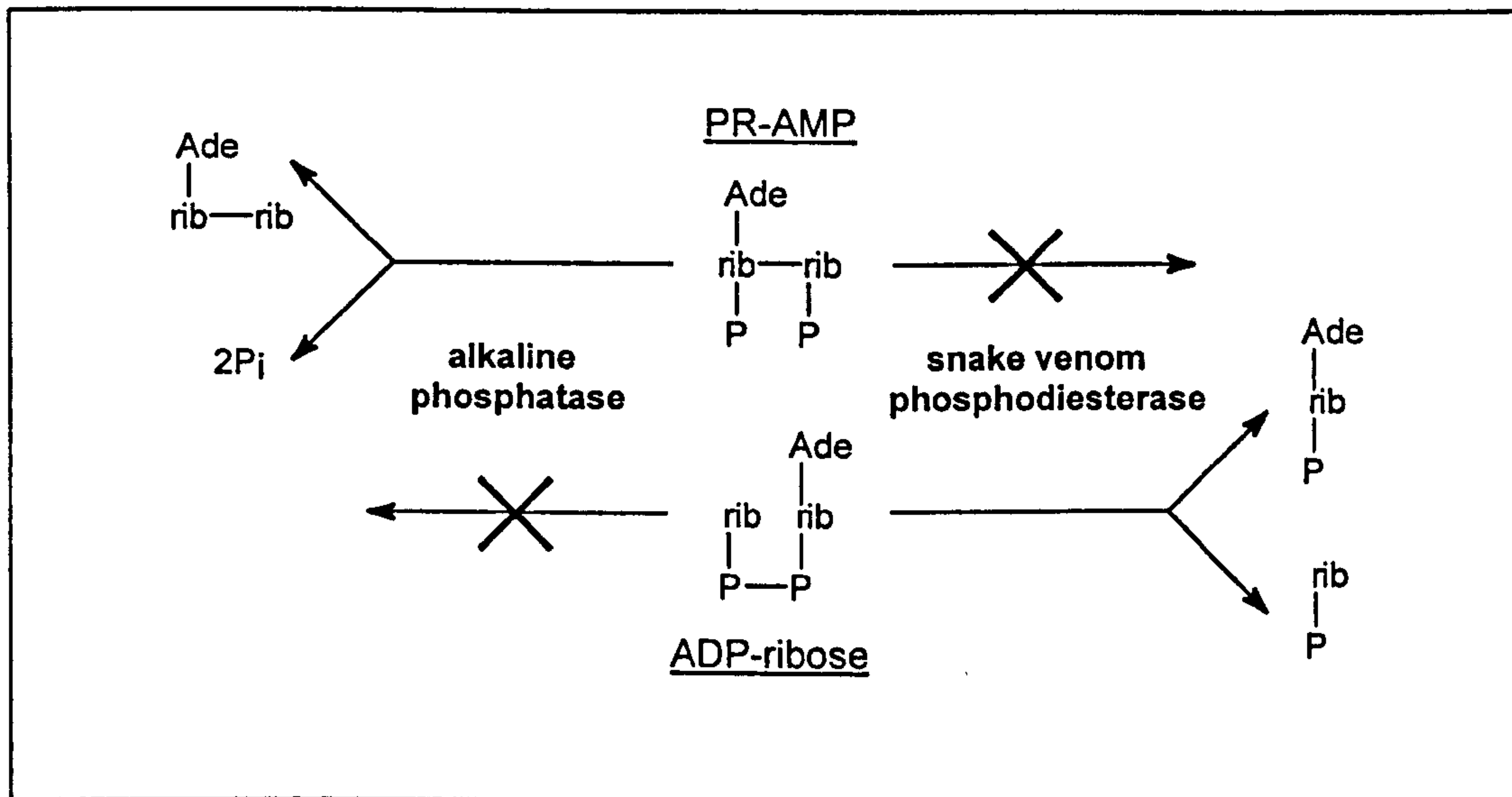
*Figure 2.7.1.1 Enzymatic hydrolysis of poly(ADP-ribose)*⁴⁹



Ade = adenosine rib = ribose P = phosphate

PR-AMP and ADP-ribose can be degraded further under different conditions: PR-AMP is cleaved by alkaline phosphatase but not cleaved by snake venom phosphodiesterase whereas the opposite is true for ADP-ribose (illustrated in *Figure 2.7.1.2*).⁴⁹

Figure 2.7.1.2 Hydrolysis of PR-AMP and ADP-ribose⁴⁹



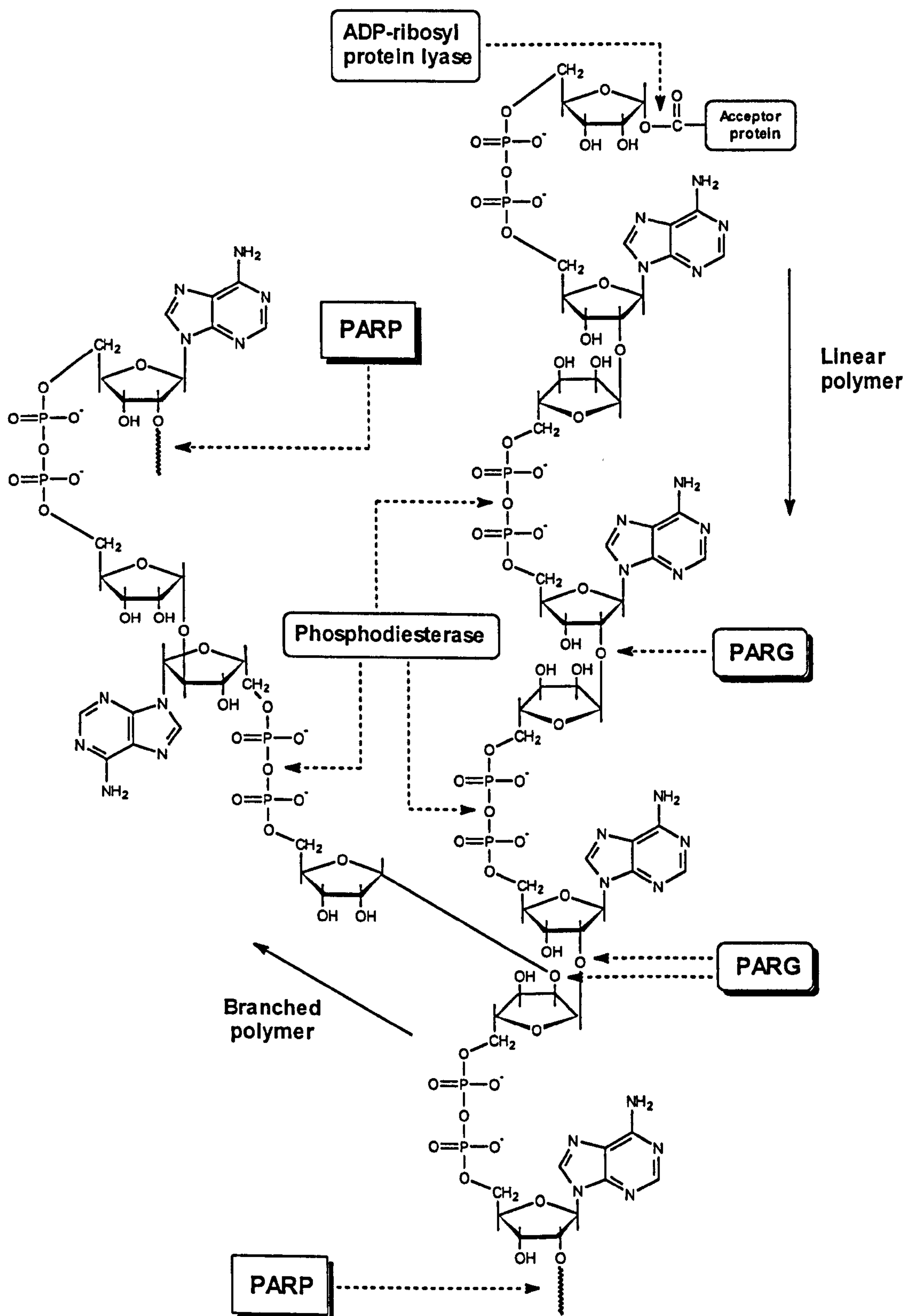
Ade = adenosine rib = ribose
P = phosphate Pi = inorganic phosphate

2.8 Summary: The Poly(ADP-ribosyl)ation System

Poly(ADP-ribosyl)ation appears to be a highly organised sequence of events. The total ADP-ribose-processing capacity of mammalian cells is impressive, with an estimated turnover of approximately 10^7 ADP-ribose residues per minute per cell for PARP and PARG, and about 72×10^6 residues for ADP-ribosyl protein lyase (assuming $\sim 10^9$ molecules of NAD^+ per cell).¹³⁰

Figure 2.8 shows a representation of the poly(ADP-ribosyl)ation system and its component enzymes.¹⁴¹ A linear polymer with a single branch point is illustrated and broken arrows indicate sites of synthesis (e.g. by PARP) or sites of cleavage (e.g. by PARG, ADP-ribosyl protein lyase or a phosphodiesterase).

Figure 2.8 The poly(ADP-ribose)ylation system¹⁴¹



Since the substrate for poly(ADP-ribosyl)ation is NAD^+ it is reasonable to assume that activation of PARP, and subsequent polymer synthesis, may influence NAD^+ metabolism. In undamaged, healthy cells, the cellular enzymatic capacity to synthesise NAD^+ exceeds normal requirements.⁴⁹ In circumstances of severe DNA damage, however, the rapid surge in poly(ADP-ribose) synthesis may result in the transient, or permanent, lowering of intracellular NAD^+ and ATP levels, which can have drastic consequences with respect to cell survival.

Attention will now be drawn to the biological consequences of poly(ADP-ribosyl)ation. Exactly what purpose does this system serve? To what extent does it influence not only NAD^+ metabolism but other biochemical processes? There have been numerous speculations on the possible roles of PARP, largely as a result of studies employing inhibitors of the enzyme. Chapter Three is concerned with literature evidence surrounding the involvement of PARP in various cellular processes.

Chapter Three: The Involvement of PARP in Cellular Processes

3.1 Methods for Studying PARP

To date, the precise biological function of PARP remains unknown. A large proportion of studies carried out rely on the use of inhibitors of the polymerase, and the evaluation of the functional consequences to the cell as a result of enzyme inhibition.

3.1.1 The Use of Chemical Inhibitors

A large number of compounds have been shown to inhibit PARP enzymatic activity both *in vitro* and *in vivo*. The most commonly used low-molecular-weight inhibitors of PARP are structurally related to the nicotinamide reaction product, and function as competitive inhibitors of the endogenous substrate, NAD^+ . The most favoured of these, 3-aminobenzamide (3AB), has served as a valuable tool in studies concerning the biological significance of poly(ADP-ribosyl)ation. However, an increasing amount of evidence indicates that, at high concentrations, 3AB and other benzamides have non-specific side effects on cellular metabolism.^{142,143} In view of this, the results of enzyme-inhibitor studies should be interpreted with caution and definitive conclusions should not be based on chemical inhibitor experiments alone. Chapter Four will discuss the development of chemical inhibitors of PARP in more detail.

3.1.2 Molecular Genetic Approaches

The advantage of using molecular genetic systems as tools for studying the role of PARP, is that the non-specific side effects associated with chemical inhibitors can be avoided. Modulation of poly(ADP-ribose) metabolism by molecular approaches has allowed the assessment of the consequences of gene disruption to living cells. Genetically modified mice which completely lack the PARP gene have been produced and now serve as

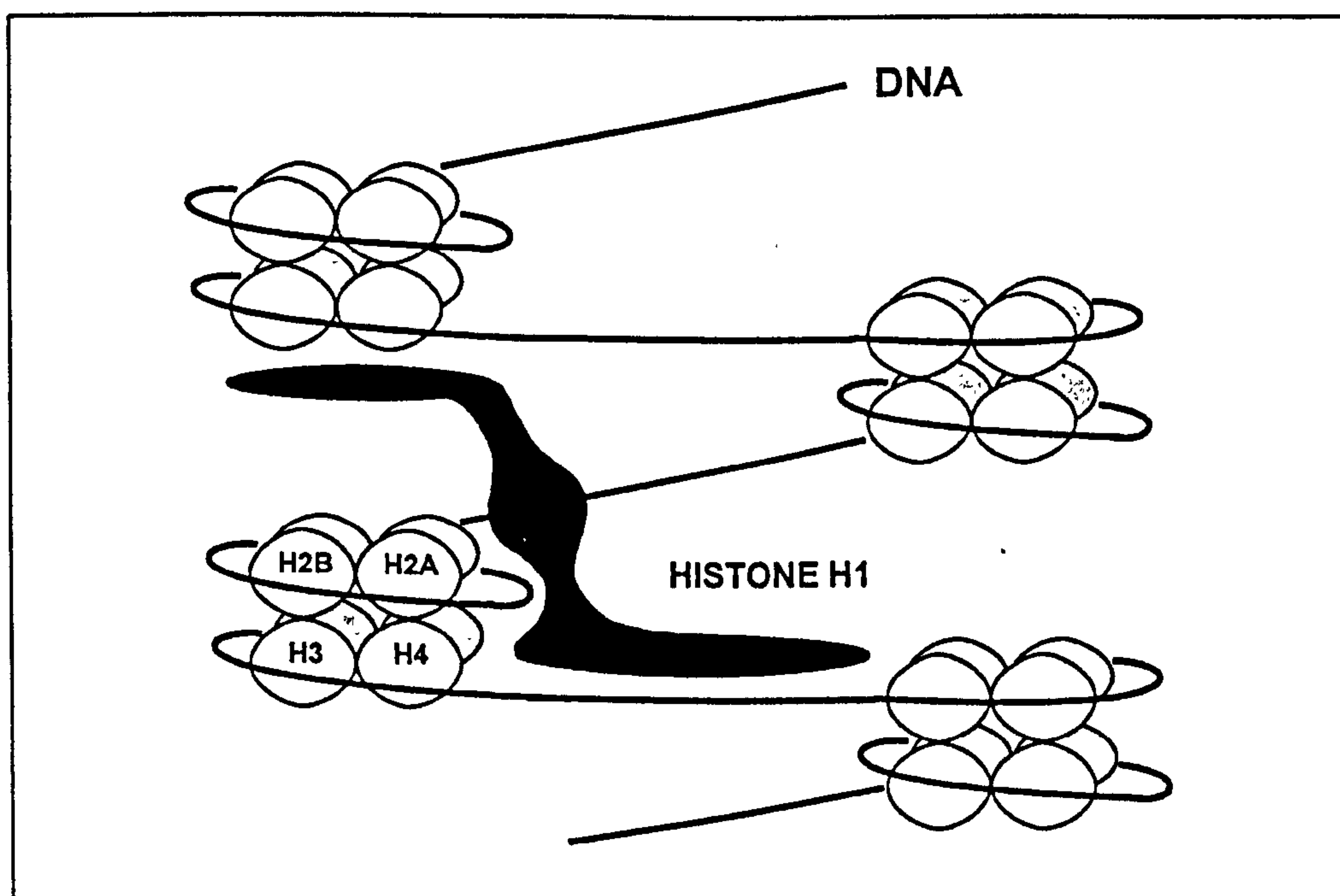
invaluable model systems for the study of PARP.^{144,145} More detailed examples of molecular genetic methods for evaluating the role of PARP will be included in the main text of the following sections.

3.2 Proposals for the Role of PARP

3.2.1 Modulation of Chromatin Structure by Poly(ADP-ribosyl)ation

The structure of chromatin largely determines the extent of DNA repair, replication and gene expression. Chromatin is composed of nucleosomal subunits which are repeating units of 167 base pairs of DNA wrapped around a core histone octamer (two each of H2A, H2B, H3 and H4).⁵⁰ These histones, classified according to their amino acid content, are either lysine-rich (H2A, H2B) or arginine-rich (H3, H4). Each nucleosome is organised into a second-order structure by interactions between histone H1 (exceptionally lysine-rich) and core histones from an adjacent nucleosome (see *Figure 3.2.1*).⁵⁰

*Figure 3.2.1 Schematic representation of chromatin structure*⁵⁰



The tight association of DNA with histones and chromosomal proteins largely affects the accessibility of DNA to enzymes or other proteins. Generally, condensed chromatin is viewed as inaccessible or only partially accessible to most nuclear processes.⁵⁰ These nuclear processes require higher-order chromatin structures, as well as individual nucleosomes, to be opened up before the DNA becomes accessible.

Ueda *et al* showed that 80-90% of PARP activity is associated with chromatin, although it is not clear exactly how polymer synthesis influences chromatin structure and its related functions.¹⁴⁶ In addition to the PARP enzyme itself, histones are the main acceptors of poly(ADP-ribose) and it has been suggested that PARP may play a role in the opening up of higher-order chromatin superstructure.¹⁴⁷ In studies carried out by Poirier *et al*, histone H1 was poly(ADP-ribosyl)ated *in vitro* and then allowed to interact with H1-depleted chromatin. It was demonstrated that even though H1 bound to chromatin, its modification by PARP resulted in the prevention of chromatin condensation into higher-order structures.¹⁴⁷ This decondensation of chromatin is reversible, however, as it was shown that incubation of decondensed poly(ADP-ribosyl)ated chromatin with PARG caused the chromatin to re-condense.^{148,149} PARG, through the degradation of polymers on H1, presumably restores histone-chromatin interactions.

According to Poirier *et al* less than 5% of the total H1 fraction is poly(ADP-ribosyl)ated but this still results in dramatic changes in chromatin structure.¹⁴⁷ The correlation between poly(ADP-ribosyl)ated H1 and decondensed chromatin was confirmed by Niedergang and coworkers.¹⁵⁰ It is interesting to note that the observations made by Poirier *et al* and Niedergang *et al* are in contrast to earlier work by Butt and coworkers, who suggested that poly(ADP-ribosyl)ation promoted chromatin condensation.¹⁵¹

3.2.2 Poly(ADP-ribosyl)ation of Nuclear Proteins

Nuclear proteins, other than histones and PARP are modified by poly(ADP-ribosyl)ation. These proteins may be involved in nuclear processes associated with chromatin. The

effects of modification on the enzymatic activities of several nuclear proteins have been investigated and the results are shown in *Table 3.1*.¹⁵²

Table 3.1 *Substrates for poly(ADP-ribose)*¹⁵²

<i>Protein</i>	<i>Consequence of poly(ADP-ribosyl)ation</i>	<i>Reference</i>
Poly(ADP-ribose) polymerase	Inhibition	153
Ca ²⁺ , Mg ²⁺ endonuclease	Inhibition	154
DNA polymerase α	Inhibition	155
DNA polymerase β	Inhibition	155
Terminal deoxynucleotide transferase	Inhibition	155
DNA ligase II	Inhibition	155
Topoisomerase I	Inhibition	156,157
Topoisomerase II	Inhibition	158
RNA polymerase II	Inhibition	159
Ribonuclease	Inhibition	160
Prolyl hydroxylase	Inhibition	161
Histone H1	Unknown	162
Histone H2B	Unknown	163
HMG proteins	Unknown	162
SV40 T antigen	Unknown	164
Adenoviral DNA binding protein	Unknown	164

Of all the enzymes which have been directly investigated, poly(ADP-ribosyl)ation results in an inhibition of activity. These include topoisomerases I and II,¹⁵⁶⁻¹⁵⁸ DNA polymerase β ^{155,165} and endonucleases.¹⁵⁴ The topoisomerases are involved in DNA replication, recombination and chromatin assembly. It has been suggested by a number

of groups that inhibition of these enzymes by poly(ADP-ribosyl)ation may serve to regulate nuclear processes which are dependent upon them. Thus, having the combined effects of inhibition of chromatin condensation and inhibition of enzymes involved in DNA synthesis and replication, under conditions of DNA damage, PARP may function to ensure that manipulation of DNA (for example, synthesis or replication) does not proceed until repair has occurred.^{50,136}

3.2.3 PARP, Chromosome Aberrations and Sister Chromatid Exchange (SCE)

In metaphase chromosomes, genetic damage is measured as chromosome aberrations and/or sister chromatid exchange (SCE).¹⁵² Chromosome aberrations generally lead to cell death, as they are a result of un-rejoined or mis-rejoined DNA double-strand breaks. The inhibition of PARP does not directly induce chromosome aberrations but it has been shown that following exposure to alkylating agents or ionising radiation, PARP inhibitors potentiate the induction of aberrations in a cell-cycle dependent manner.^{152,166}

The mechanism of SCE formation is not understood but inhibitors of PARP have been shown to increase SCE frequency in a dose-dependent manner (the more potent the inhibitor, the more SCE's are induced).^{152,167-169} Despite the many conflicting reports about the involvement of PARP in cellular responses to DNA damage, the increase in SCE frequency appears to be the one agreed-upon cellular consequence of enzyme inhibition.¹⁵²

3.2.4 Poly(ADP-ribosyl)ation and Gene Amplification

Gene amplification in normal mammalian cells is rare but represents a mechanism by which tumour cells may increase levels of specific proteins and thereby become resistant to certain agents.¹⁵² Gene amplification induced by carcinogens has been studied. Bürkle *et al* showed that methotrexate-resistance and dihydrofolate reductase gene amplification were increased when PARP was inhibited by 3AB in cell culture systems.

The conclusion of these results was that PARP may act as a negative regulatory factor in the induction of gene amplification.¹⁷⁰

The role of PARP in the induction of viral replication and amplification has also been investigated. Bürkle *et al* reported the enhanced amplification of integrated simian virus 40 (SV40) sequences in SV40-transformed Chinese hamster CO60 cells, which had been treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and 3AB.¹⁷¹ Supportive data were presented by Ding and Smulson, who observed enhanced gene amplification in cells depleted of PARP mRNA by antisense RNA expression.¹⁷²

3.2.5 Poly(ADP-ribosyl)ation and DNA Repair

Since the observation, made by Durkacz *et al* in 1980, that low-molecular-weight chemical inhibitors of PARP caused a delay in strand-break rejoining, PARP has been associated with the DNA repair process.¹⁷³ Its participation in DNA repair has, however, remained controversial, largely due to the lack of positive evidence to support the proposal. The following sections will discuss proposed models for the role of PARP in cellular defence mechanisms.

3.2.5.1 Histone Shuttle Mechanism

The histone shuttle mechanism, put forward by Althaus and coworkers, represents a model for the involvement of PARP in DNA excision repair.¹³⁰ The covalent linkage between PARP and acceptor proteins is generally considered to be a pre-requisite for enzymatic activity, but Althaus *et al* suggest that PARP may function primarily *via* non-covalent interactions with nuclear proteins.¹³⁰

The automodification reaction of PARP has been found to follow a processive reaction mode *in vitro*.¹⁷⁴ This processive nature has also been observed in more complex systems, such as in isolated nuclei, nucleosomal core particles and intact cells, although the polymer patterns identified *in vivo* differ from those produced *in vitro*.¹⁷⁵⁻¹⁷⁷ Histones

H1, H2A, H2B, H3 and H4 have been identified as key regulatory factors determining the numbers, sizes and branching frequencies of polymers.¹⁷⁵ Naegeli and Althaus demonstrated that when PARP encountered DNA-bound histones *in vitro*, it had the adaptability to produce histone-type specific polymer patterns which were almost identical to those produced *in vivo*.¹⁷⁵ They concluded that histones may directly influence the polymer termination reaction of PARP, although the relevance of these histone-specific adaptations *in vivo* is unclear.

Investigations of the interactions between histones and ADP-ribose polymers revealed that the mode of binding was stronger and more specific than would be expected on the basis of electrostatic interactions alone.¹⁷⁸ Panzeter *et al* used highly stringent assay conditions, to eliminate non-specific electrostatic interactions, and investigated the non-covalent binding affinities of various proteins towards different molecular classes of ADP-ribose polymers.¹⁷⁸ They found that of all the proteins tested, only histones H1, H2, H3, H4 and protamine (which replaces histones in the final stages of spermatogenesis) bound to the polymers. It is interesting to note that the primary site of polymer binding of histone H1 is the carboxyl-terminal domain¹⁷⁸ which is also the domain that is most effective in inducing higher order chromatin structure.¹⁷⁹

The polymer-histone complexes were found to exhibit unusually high binding strengths and were resistant to high salt concentrations, detergent and strong acids.¹⁷⁸ The interactions between different histone and polymer types were also found to have specificity: branched polymers were the most highly preferred binding counterparts, followed by long and short linear polymers, respectively.¹⁷⁸ The hierarchy of histone binding was established as $H1 > H2A > H2B = H3 > H4$.¹⁷⁸

In agreement with other suggestions that PARP is somehow involved in nucleosomal unfolding, it has been shown that automodified PARP can cause the dissociation of histones from a core DNA fragment *in vitro*.¹⁸⁰ This dissociation is prevented in the presence of competitive PARP inhibitors. The negatively charged, enzyme-bound polymers are more acidic than DNA and would, therefore, be expected to compete with

DNA for histone binding. Realini and Althaus demonstrated that automodified PARP was 10-fold more potent in dissociating histone H2B from H2B-DNA complexes when compared to free polymers. They also showed that the branch points of ADP-ribose polymers had the highest binding affinities for histones and suggested that the polymers of automodified PARP may selectively target histones for dissociation from DNA.¹⁸¹

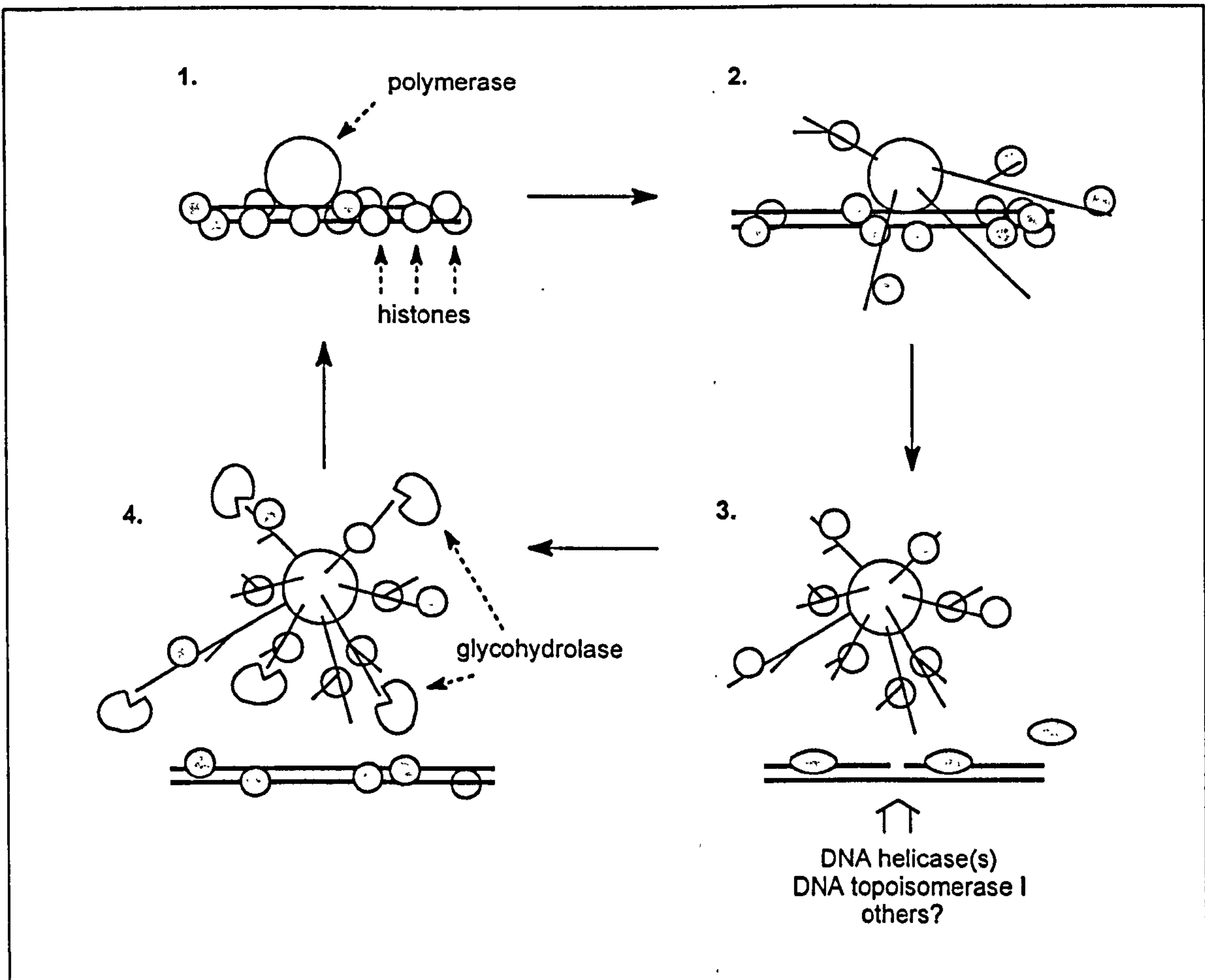
The reverse effect *i.e.* the reassociation of histone-DNA complexes, is mediated by PARG.¹⁸¹ Briefly, polymers are initially degraded by a combination of endo- and exoglycosidic actions and branched polymers are degraded more slowly than linear polymers. The avid binding of histones to branch sites has been suggested as the reason for the slower degradation of branched polymers.¹⁸¹

Thus, to summarise, the histone shuttle mechanism is thought to operate in a processive, adaptive, target-specific and reversible fashion.¹⁸² The shuttling of histones on and off DNA, effected by PARP and PARG, is proposed to influence chromatin condensation and allow enzymes or proteins involved in nuclear processes to gain access to the DNA.¹⁸³ *Figure 3.2.5.1* shows a schematic representation of the histone shuttle mechanism.¹⁸³

Although the histone shuttle mechanism has been demonstrated *in vitro*, evidence for *in vivo* histone shuttling must also be considered. Processivity, adaptiveness and reversibility of the shuttle mechanism have been observed *in vivo* and depletion of PARP has been shown to block nucleosomal unfolding in mammalian cells.¹⁸⁴ Thus, it may be possible that PARP is involved in the dissociation of histone-DNA complexes.

However, several features of poly(ADP-ribosyl)ation cannot be explained by the histone shuttle mechanism. The most striking example is the presence of the poly(ADP-ribosyl)ation system in dinoflagellates despite the fact that these primitive eukaryotes completely lack histones in their cell nuclei.⁵⁸ Therefore, the involvement of PARP in the histone shuttle mechanism should be viewed with caution, as the enzyme may have a role other than or beyond this process.

Figure 3.2.5.1 Schematic representation of the histone-shuttle mechanism¹⁸³



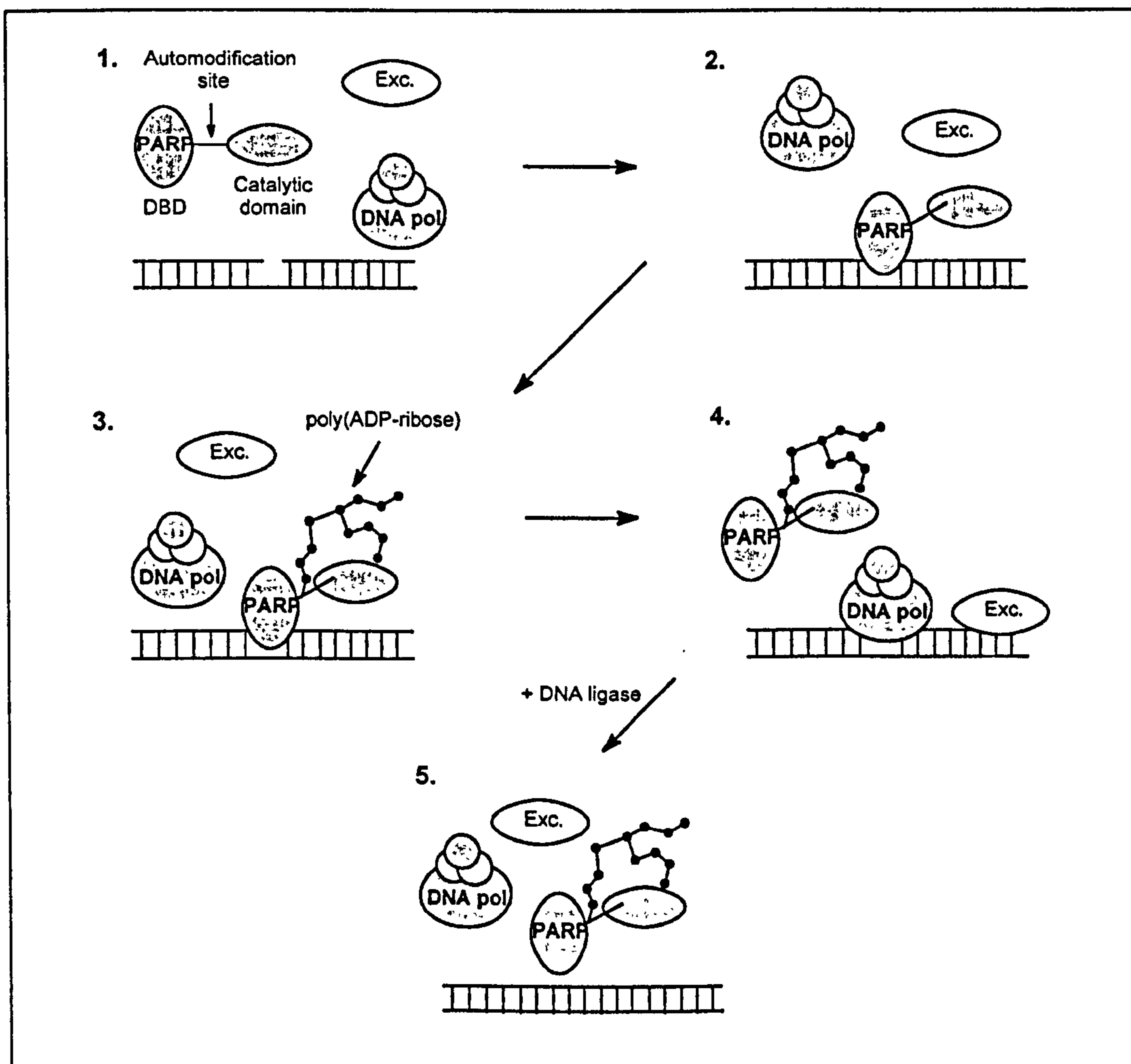
- 1 PARP binds to a DNA strand break
- 2 automodified PARP causes the dissociation of histones from DNA
- 3 dissociation of the modified enzyme allows access for other enzymes
- 4 the polymers are degraded by PARG and the histones reassociate with the DNA

3.2.5.2 Non-Histone Mechanism

The role proposed by Satoh and Lindahl (see *Figure 3.2.5.2*,¹⁸⁵) for poly(ADP-ribose) formation in the DNA repair process, essentially contradicts the histone shuttle mechanism. Using histone-depleted cell extracts, Satoh and Lindahl demonstrated that inhibition of PARP by 3AB resulted in interference with the ability of the cells to repair

γ -irradiation-induced DNA damage. They suggested that rather than carrying out a positive function in DNA repair, PARP may have an equally as important negative function to fulfil.

Figure 3.2.5.2 *Proposed model for the involvement of PARP in DNA repair*¹⁸⁵



- 1 when a strand break is introduced, PARP, excision enzymes (Exc.) and DNA polymerases (DNA pol.) compete for binding to the break.
- 2 DNA-bound PARP limits access to the strand break for repair enzymes
- 3 automodification occurs once PARP is bound to the break
- 4 automodified PARP dissociates from the break and allows access for repair enzymes
- 5 excision-repair seals the DNA strand break.

Whilst the histone-free cell extracts used by Satoh and Lindahl refute the histone shuttle mechanism, they agree that heteromodification of histone H1 in chromatin may aid chromatin decondensation. Again, their proposal relies on the importance of accessibility of the DNA strand breakage to repair enzymes, which is achieved when the DNA-bound polymerase is automodified and subsequently dissociates from the DNA. However, according to Satoh and Lindahl, the build-up of polymers during automodification may serve to prevent biologically inappropriate side reactions from occurring, such as DNA recombination. Inappropriate DNA recombination may lead to chromosomal aberrations which would be detrimental to the cell, and PARP may thus, have a 'protective' role. Satoh and Lindahl's proposal concerning PARP and the prevention of homologous recombination is supported by Chatterjee and Berger.¹⁸⁶

Alternatively, Satoh and Lindahl raised the possibility of a signal mechanism perhaps elicited by the PARP enzyme, once bound to a DNA break. This signal may negatively regulate DNA synthesis and replication to ensure that unrepaired DNA is not duplicated.¹⁸⁵ It must be noted, however, that there is no positive, direct evidence for either the prevention of DNA recombination or the signalling mechanism.

3.2.5.3 Poly(ADP-ribosylation) and Ligation of DNA

The DNA repair process can be broadly divided into four stages:⁴⁹

- i) incision (at or near the DNA lesion),
- ii) removal of damaged/defective nucleotides and some adjacent DNA,
- iii) resynthesis, to fill the gap (unscheduled DNA synthesis: UDS),
- iv) religation to seal the DNA.

PARP inhibitors do not affect incision events, which suggests that the active enzyme is not required for the early stage of DNA repair.¹⁸⁷ The rate of removal of damaged DNA is also unaffected by inhibition of PARP.¹⁸⁷ The effects of PARP inhibitors on UDS have been determined by a number of research groups.¹⁸⁸⁻¹⁹⁰ In most cases, PARP inhibitors

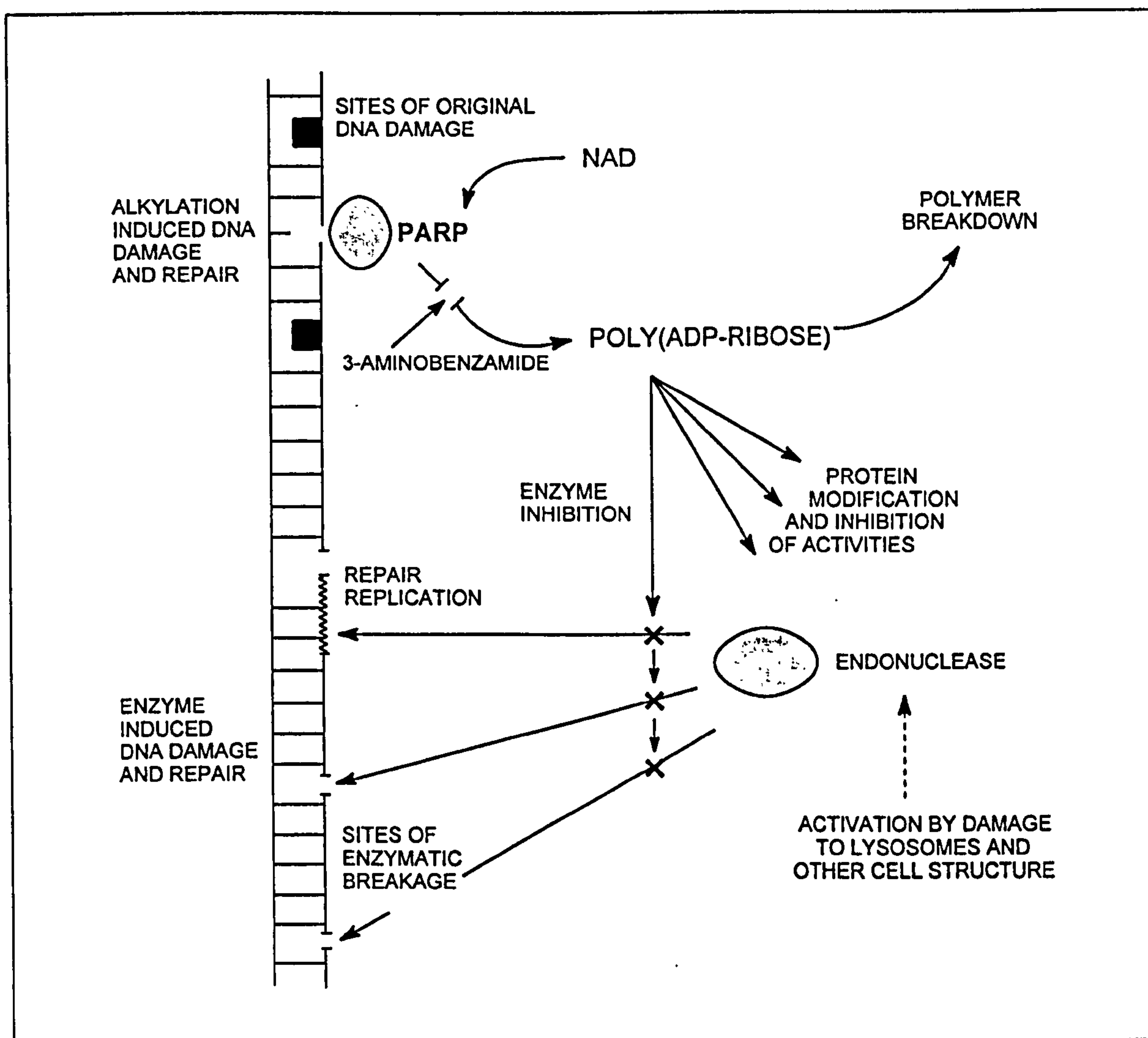
did not block UDS and, in fact, stimulated UDS depending on the conditions and inhibitors used.

Based on the above data, Creissen and Shall proposed that PARP was involved at or near the ligation step of DNA repair.¹⁹¹ Mammalian cells contain DNA ligase I and II, both of which are thought to be involved in DNA repair.¹⁸⁷ Creissen and Shall found that when L1210 murine leukaemia cells were treated with the alkylating agent dimethyl sulfate (DMS), the overall DNA ligase enzyme activity in the cells was increased. This increase in ligase activity was prevented by inhibition of PARP.¹⁹¹

It should be noted, however, that Yoshihara *et al* found that DNA ligase II activity was inhibited by poly(ADP-ribosyl)ation.¹⁵⁵ In view of these findings, Cleaver and Morgan proposed an entirely different model for the role of PARP within the cell.¹⁹² They discarded the notion of DNA ligase II regulation by PARP and focussed on the poly(ADP-ribosyl)ation of enzymes which are normally activated on exposure to alkylating agents. Since the poly(ADP-ribosyl)ation of most nuclear proteins results in their inhibition, Cleaver and Morgan suggested that inhibition of PARP may lead to chaotic, non-specific attack of DNA by nuclear enzymes such as Ca^{2+} - and Mg^{2+} -dependent endonucleases. Random nuclease attack on DNA would create additional sites of damage, which would lead to increased DNA damage and cell killing but would not be expected to lead to a slower rate of repair. The authors directly measured the rate of ligation during DNA repair when PARP was inhibited. The rate was found to be unchanged when compared to control cells, and it was concluded that poly(ADP-ribosyl)ation did not regulate the ligation step of DNA repair.

The pathway proposed by Cleaver and Morgan is shown in *Figure 3.2.5.3*.¹⁹² This model views the process of poly(ADP-ribosyl)ation as a mechanism by which the overall level of enzyme activity is lowered in cells which are exposed to DNA-damaging agents.¹⁹²

Figure 3.2.5.3 Proposed model for the role of PARP in damaged cells¹⁹²

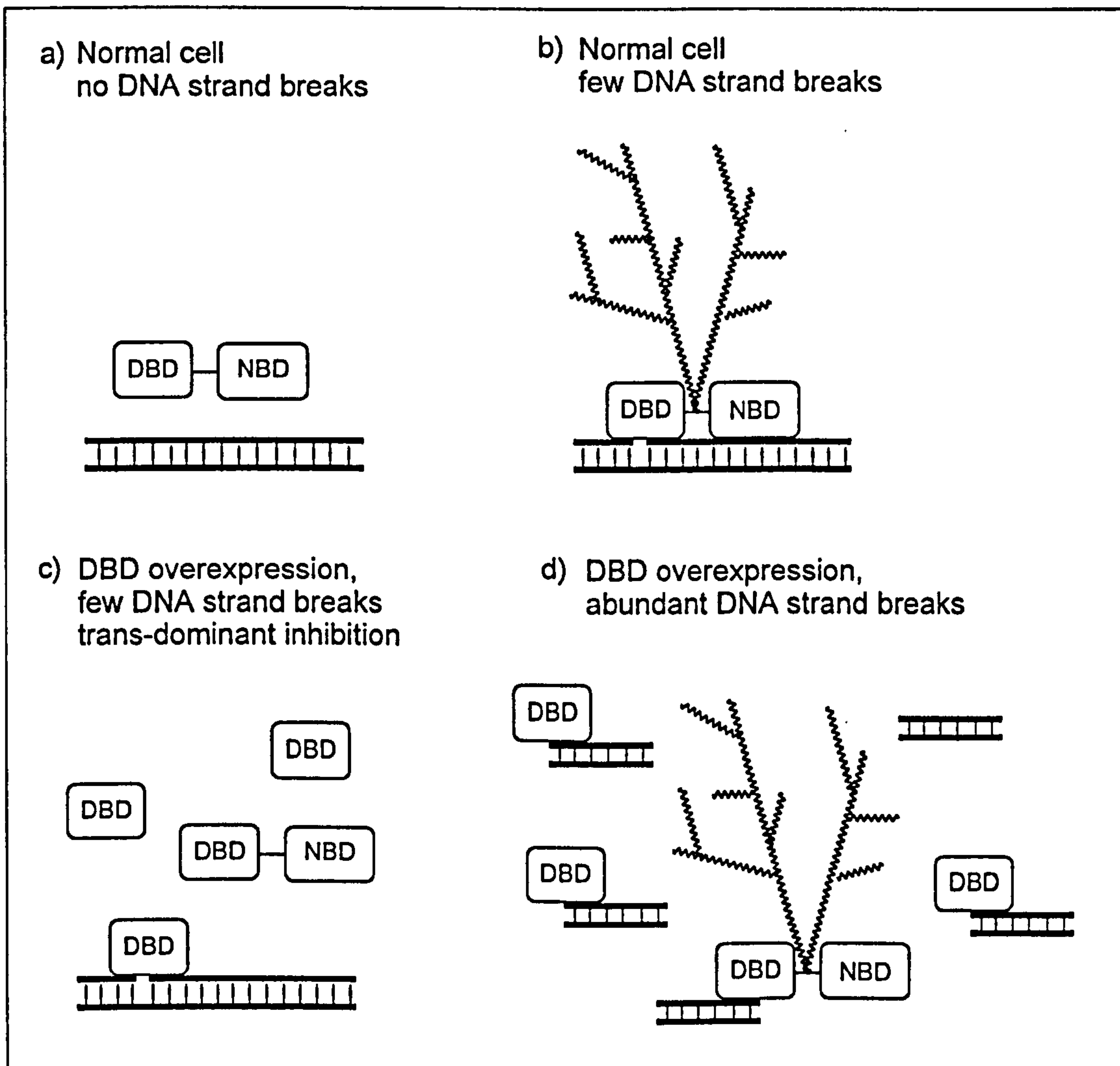


3.2.5.4 PARP and Excision Repair

The participation of PARP in the processes of NER and BER has been evaluated using molecular genetic techniques.^{193,194} Küpper *et al* demonstrated that the endogenous activity of human PARP can be inhibited by the overproduction of its own DNA-binding domain (DBD).¹⁹³ The 42kDa DBD acts as a dominant negative version of the enzyme and leads to transient inhibition of resident PARP. This is the result of competition for

DNA strand breaks between endogenous PARP and the overproduced DBD. *Figure 3.2.5.4 depicts the basic principle of trans-dominant inhibition.*¹⁹⁵

Figure 3.2.5.4 Principle of trans-dominant inhibition of poly (ADP-ribose) polymerase¹⁹⁵



DBD = DNA-binding domain

NBD = nicotinamide-binding domain

- a) PARP does not bind to DNA in undamaged cells
- b) if a break is present, PARP binds to it and automodification occurs
- c) *trans*-dominant inhibition of PARP occurs due to competing resident PARP and DBD molecules
- d) the inhibition effect is abrogated when an excess of DNA breaks is present.

Thus, using a dominant negative version of human PARP has the effect of transient inhibition and is essentially equivalent to using low molecular-mass chemical inhibitors of PARP. In order to evaluate the role of PARP in BER and NER, the consequences on DNA repair of exposure of human fibroblasts and CV-1 monkey cells to MNNG and UV radiation, was assessed.¹⁹⁴ Damage induced by treatment with the alkylating agent MNNG would mainly be removed by the BER process, whereas UV radiation-induced DNA damage would be eliminated by NER (see section 1.1.1 for more detail). Molinete *et al* observed the *trans*-dominant inhibition of PARP and the inhibition of UDS in MNNG-treated cells. However, in UV radiation-treated cells, no such inhibition was noted.¹⁹⁴ These observations point to a role for PARP in the BER process but not in NER.

3.2.6 PARP and DNA Replication

PARP has been shown to modify the catalytic activities of various replicative enzymes (see *Table 3.1*). These include DNA polymerase α , DNA polymerase β , terminal deoxytransferase, DNA ligase II, topoisomerases I and II and Ca^{2+} - and Mg^{2+} -dependent endonuclease.

DNA polymerase α is essential for the initiation of cellular DNA replication.¹⁹⁶ It has been suggested by Eki, that since poly(ADP-ribosyl)ation of DNA polymerase α inhibits its activity, the initiation of DNA replication may be negatively regulated by PARP.¹⁹⁷ Other reports have indicated that unmodified PARP directly associates with DNA polymerase α and that at high levels of PARP activation, the activity of DNA polymerase α is stimulated.^{198,199} The association of the two polymerases was demonstrated by their co-immunoprecipitation, using antibodies directed at DNA polymerase α , suggesting that the enzymes may be part of a complex.¹⁹⁸ The above findings suggest that PARP and poly(ADP-ribose) may positively *and* negatively regulate DNA replication, respectively.^{198,199}

PARP has been shown to copurify with multiprotein DNA replication complexes (MRCs) which contain DNA polymerases α and β , DNA primase, DNA ligase, DNA helicase and topoisomerases I and II.²⁰⁰ PARP has also been observed to copurify with DNA fragments enriched with replication forks.¹¹⁷ Thus, PARP may control the progression of the replication fork under conditions of DNA damage to prevent replication occurring prior to repair.²⁰⁰

3.2.7 PARP and Cell Death

Cell death may occur by either one of two pathways: necrosis or apoptosis.²⁰¹ Necrosis generally follows physical injury to the cell and is independent of genetic control. Apoptosis, or programmed cell death, is triggered by developmental or environmental stimuli. It is a deliberate, genetically controlled cellular response involving a series of specific events that culminate in the death of the cell. Apoptosis is of fundamental importance to normal development and dysregulation of the process is associated with an increased susceptibility to cancer.²⁰¹ There is evidence to show that cells derived from human cancers may have a decreased ability to undergo apoptosis, and that this defect could be involved in the aberrant survival or development of cancer cells.^{202,203}

Both necrosis and apoptosis are characterised by specific morphological changes. Necrotic cells display cytoplasmic organelle destruction whereas apoptosis is typified by nuclear condensation and DNA fragmentation, resulting from the degradation of the internucleosomal linker regions.²⁰⁴ One of the main distinguishing features of apoptosis compared to necrosis is that the efficient and complete elimination of the cell results in minimal damage to surrounding tissues and the absence of an inflammatory response.²⁰¹ Apoptosis can also be thought of as a protective mechanism, especially in instances where cells are subjected to a substantial amount of irreparable DNA damage. Elimination of such extensively damaged cells *via* apoptosis would thus protect a multicellular organism against the accumulation of cells, which may ultimately be detrimental to the survival of that organism.

A large number of genes are involved in the regulation of apoptosis and, broadly speaking, the genes involved are suppressors/promoters or effectors of apoptosis. Suppressors of apoptosis include bcl-2, a 26 kDa protein which prevents a number of types of apoptosis in various cell lines, including apoptosis induced by cancer chemotherapeutic agents.²⁰⁵ Promoters of apoptosis include other members of the bcl-2 gene family, such as bax²⁰⁶ and bak.²⁰⁷ The most important effectors of apoptosis are members of the interleukin-1 β -converting enzyme (ICE) family of cysteine proteases.²⁰⁸ A number of ICE-related proteases involved in apoptosis have been identified in mammals and the many observations that inhibitors of ICE family members inhibit apoptosis, point to essential roles for the ICE proteases in the regulation and implementation of cell death. Active ICE proteases cleave cellular proteins and this cleavage of critical substrates is thought to produce the apoptotic phenotype.²⁰⁸

Another prominent feature of apoptosis is the cleavage of the PARP enzyme. This is effected by CPP32 β /apopain, an ICE family protease.²⁰⁹ In many forms of apoptosis, including those induced by chemotherapeutic agents, PARP is specifically cleaved to give an approximately 85 kDa fragment containing the automodification and catalytic domains, and an approximately 31 kDa fragment containing the DBD.²¹⁰ Cleavage of PARP occurs at a site within the NLS which results in the loss of nuclear retention for the larger apoptotic fragment.²¹¹ The appearance of these fragments has become a hallmark of the apoptotic process and has provided a sensitive marker for its detection.^{212,213} It was recently suggested that the degradation of PARP may also provide a sensitive marker for the detection of necrotic cell death, as it was observed that cleavage patterns for PARP during necrosis and apoptosis are different in human leukaemia cells.²¹³

Activation of PARP in heavily damaged cells can result in the substantial depletion of intracellular NAD⁺ and ATP levels,²¹⁴ and has been shown to be related to cell lysis.²¹⁵ Apoptosis requires NAD⁺ and ATP as energy sources and, since PARP uses NAD⁺ as a substrate, inhibition or apoptotic inactivation of the polymerase would be expected to preserve intracellular NAD⁺ and ATP pools, and could be a reason for the cleavage of PARP during apoptosis. In some experimental systems, 3AB and its analogues have

been shown to 'rescue' cells from death, suggesting that PARP has a critical role in apoptosis.²¹⁶⁻²¹⁸

3.2.8 Poly(ADP-ribosyl)ation and the Cell-Cycle Checkpoint

The involvement of PARP in the progression of the cell cycle has been investigated using inhibitor experiments. A variety of cellular responses to DNA damage can be observed in the cell, including transient cell-cycle arrest in G1 and G2 phases.²¹⁹ G1 arrest prevents replication of the damaged DNA template and G2 arrest prevents the segregation of damaged chromosomes. Defects in cell-cycle checkpoint mechanism are thought to be involved in cell death, apoptosis, genetic instability and carcinogenesis.²²⁰

In studies carried out by Nozaki *et al*, following γ -irradiation, inhibitors of PARP were found to suppress G1 arrest in murine 3T3-a cells.²²¹ Although the exact molecular mechanism of the G1 arrest pathway has not yet been clarified, suppression of the G1 arrest by PARP inhibitors indicates that G1 arrest may be dependent on poly(ADP-ribosyl)ation. On the other hand, G2 arrest was found to be enhanced and prolonged in the presence of PARP inhibitors and it was suggested that PARP is not critical for the induction of G2 arrest but may be involved in the regulation of G2 arrest release.^{221,222}

3.2.9 Poly(ADP-ribose) Activity and HIV-1 Infection

Apoptotic cell death, following persistent HIV-1 infection is thought to be the most important feature of AIDS pathogenesis.²²³ Due to the involvement of PARP in apoptosis, the relationship between the enzyme and HIV-1 infection has been investigated by Tanaka *et al*.²²⁴

Nuclear extracts from cells infected with HIV-1 were found to have a decreased level of PARP activity resulting from a reduction of levels of the enzyme itself rather than from inhibition or modification of its activities.²²⁴ The researchers suggested that low levels of PARP in infected cells may be necessary for the effective production of the virus and/or

for a stable virus/host interaction. Thus, HIV-1 may elicit some kind of mechanism to decrease the levels of PARP in host cells and prevent them from entering the apoptotic pathway, as a means of ensuring the survival of the virus.²²⁴

3.3 The Use of Transgenic Model Systems

Several DNA-repair-deficient model systems are currently available, which allow the characterisation and evaluation of DNA-repair-deficient syndromes. The development of transgenic animal technology and especially the targeting and manipulation of genes, now offers new means for the *in vivo* analysis of DNA repair and cell-cycle control mechanisms.

Transgenesis is the generation of genetically modified animals by the introduction of genes into the germ line.²²⁵ The process of homologous recombination allows for the precise targeting of a given gene. Murine embryonic stem (ES) cells can contribute to the germ cell lineage and, as such, are commonly used as vehicles for introducing heritable changes in mice.²²⁵ ES cells can be used to produce mouse strains which are heterozygous for a specific gene. These animals can subsequently be bred to obtain mice which are homozygous for the targeted gene. Thus, the functional consequences of *in vivo* gene disruption can be assessed in model systems with absent genes: such systems are known as 'knockout' model systems.^{225,226}

3.3.1 The Development of PARP 'Knockout' Mice

Due to the absence of the *PARP* gene in *Saccharomyces cerevisiae* (yeast), a convenient, simple genetic system for the study of this particular gene has thus far been unavailable. Gene targeting and homologous recombination have been used to develop animal and cellular model systems which completely lack the *PARP* gene.^{144,145} The results have been used to assess the *in vivo* role of the PARP enzyme. It must be noted, however, that different views have been put forth concerning the involvement of PARP in cellular processes. These probably stem from the mutational differences in the genes, as one set

of mutants are lacking in part of the first zinc finger, f I,¹⁴⁴ whereas the other mutants lack the entire f I and part of the second finger f II.¹⁴⁵

De Murcia *et al* inactivated both alleles of the *PARP* gene by gene targeting in mice.¹⁴⁴ These PARP knockout mice (PARP $-/-$ mice) were viable and fertile, but exhibited a marked sensitivity towards DNA-damaging agents, such as γ -irradiation and *N*-methyl-*N*-nitrosourea (MNU). Wang *et al* generated PARP $-/-$ mice by homologous recombination and produced mutant mice which were also viable, apparently healthy and fertile.¹⁴⁵ These mice, however, were found to develop pathological skin aberrations 5-6 months after birth. It was suggested that since the skin is constantly exposed to environmental stress, the lack of the PARP enzyme in keratinocytes would not allow the elimination of cells containing damaged DNA. Thus, the cells may be susceptible to neoplastic transformation. Wang *et al* hypothesised that PARP somehow interferes with cell-cycle regulation specifically in keratinocytes. Further results demonstrated that PARP was not required for normal cell differentiation and mouse development.²²⁷

In order to test the efficiency of DNA-repair in PARP $-/-$ cells, Auer *et al* measured the recovery of PARP $-/-$ cells after treatment with DNA-damaging agents.²²⁷ They concluded that PARP was not directly involved in BER or NER, which is in contrast to the results of Molinete *et al*¹⁹⁴ but in accordance with the *in vitro* model put forth by Satoh *et al*.²²⁸

3.4 PARP and Carcinogenesis

Numerous studies on animals and cell cultures have shown that PARP inhibitors potentiate the cytotoxicity of DNA-damaging agents. However, variations in results have served to create confusion, as PARP inhibitors have been shown to both increase and decrease the transformation frequency of cultured cells.⁵⁰

3.4.1 The Relevance of PARP Inhibitors as Clinical Agents

The inhibition of PARP is thought to lead to a decrease in the repair rate of DNA lesions. Thus, due to the rapid growth of cancer cells, the combined action of PARP inhibitors with DNA-damaging agents, compared to the effects of the DNA-damaging agents alone, may be the preferential killing of cancer cells over normal cells, by the blockage of DNA repair.¹⁴¹ However, the inhibition of repair in normal cells after treatment with PARP inhibitors and cytotoxic agents may result in an increase in transformation frequency.¹⁵² Nevertheless, PARP inhibitors have been shown to potentiate the effects of a variety of DNA damaging drugs and, as such, have potential application as therapeutic agents, to be used in conjunction with currently used anticancer treatments. Chapter Four is concerned with the development of chemical inhibitors of PARP.

3.5 Summary

PARP is a truly fascinating enzyme, whose role in cellular defence mechanisms remains unclear, despite the numerous investigations undertaken. The speculation that PARP facilitates chromatin condensation, along with the regulation of nuclear proteins involved in DNA synthesis and replication, is an attractive proposal for a protective role of the enzyme in the nucleus. Decondensation of chromatin would thus allow nuclear processes, such as DNA repair, to proceed and recondensation of the chromatin would be effected by PARP.

This modulation of chromatin structure is largely dependent on the poly(ADP-ribosyl)ation of histones, especially histone H1, which is involved in higher-order chromatin superstructure. The histone shuttle mechanism for DNA excision repair, proposed by Althaus and coworkers,¹³⁰ incorporates this aspect of poly(ADP-ribosyl)ation and although this proposal does appear possible, the model put forward by Satoh and Lindahl indicates that histone modification by PARP is not of primary importance.¹⁸⁵

The function of PARP according to Satoh and Lindahl, is to prevent inappropriate DNA recombination from occurring, an event which, if uncontrolled, could lead to an increase in chromosomal aberrations and ultimately cell death. Although there is no conclusive evidence to support this proposal, taken together with data from Wiencke and Morgan¹⁶⁶ suggesting that the inhibition of PARP potentiates the induction of chromosomal aberrations, this proposal appears credible.

The relationship between PARP and DNA ligase II, originally proposed by Creissen and Shall¹⁹¹ was refuted by Yoshihara *et al*¹⁵⁵ and Cleaver and Morgan.¹⁹² The latter authors proposed an alternative role for PARP during cytotoxic events, and suggested that the potentiation of cell killing by inhibitors of PARP was due to the activation of non-specific intracellular endonucleases and not to the regulation of DNA repair.

In studies using the DBD of PARP as a *trans*-dominant inhibitor, PARP has been associated with the BER process,¹⁹⁴ although recent results obtained from genetically engineered PARP *-/-* cells imply that PARP is neither associated with BER nor NER.²²⁷ It was hoped that the production of genetically modified animals might serve to clarify the role of the PARP enzyme *in vivo*. In fact, the construction of PARP *-/-* mice has offered little new positive information. Despite the many proposals put forth for the role of PARP in the DNA repair process, the viability of the PARP *-/-* mice confirms that normal DNA repair occurs in the absence of PARP,¹⁴⁴ However, as is in keeping with the role proposed by Satoh and Lindahl,¹⁸⁵ in the absence of PARP, there may be an increase in chromosomal aberrations due to inappropriate homologous recombination. This may manifest as pathological disorders in older animals, and indeed, Wang *et al* detected skin aberrations in PARP *-/-* mice 5-6 months after birth.¹⁴⁵

The role of PARP in the apoptotic process remains to be clarified. The significance of the degradation of PARP and its contribution to the apoptotic cascade of events is unclear, although PARP cleavage itself represents a sensitive marker for the detection of cell death.^{212,213} The decreased levels of the PARP enzyme in persistently infected HIV-1 cells may represent a new target for the study of the disease.^{223,224}

Whatever the mode of action of PARP, the fact remains that inhibitors of the enzyme are known to potentiate the effects of DNA damaging agents. This activity confers potential value to the inhibitors as resistance-modifying agents. Thus, PARP inhibitors can be used as adjuncts to cancer treatment, in combination with DNA-damaging agents or radiation.

Chapter Four: The Development of Chemical Inhibitors of PARP

4.1 Introduction

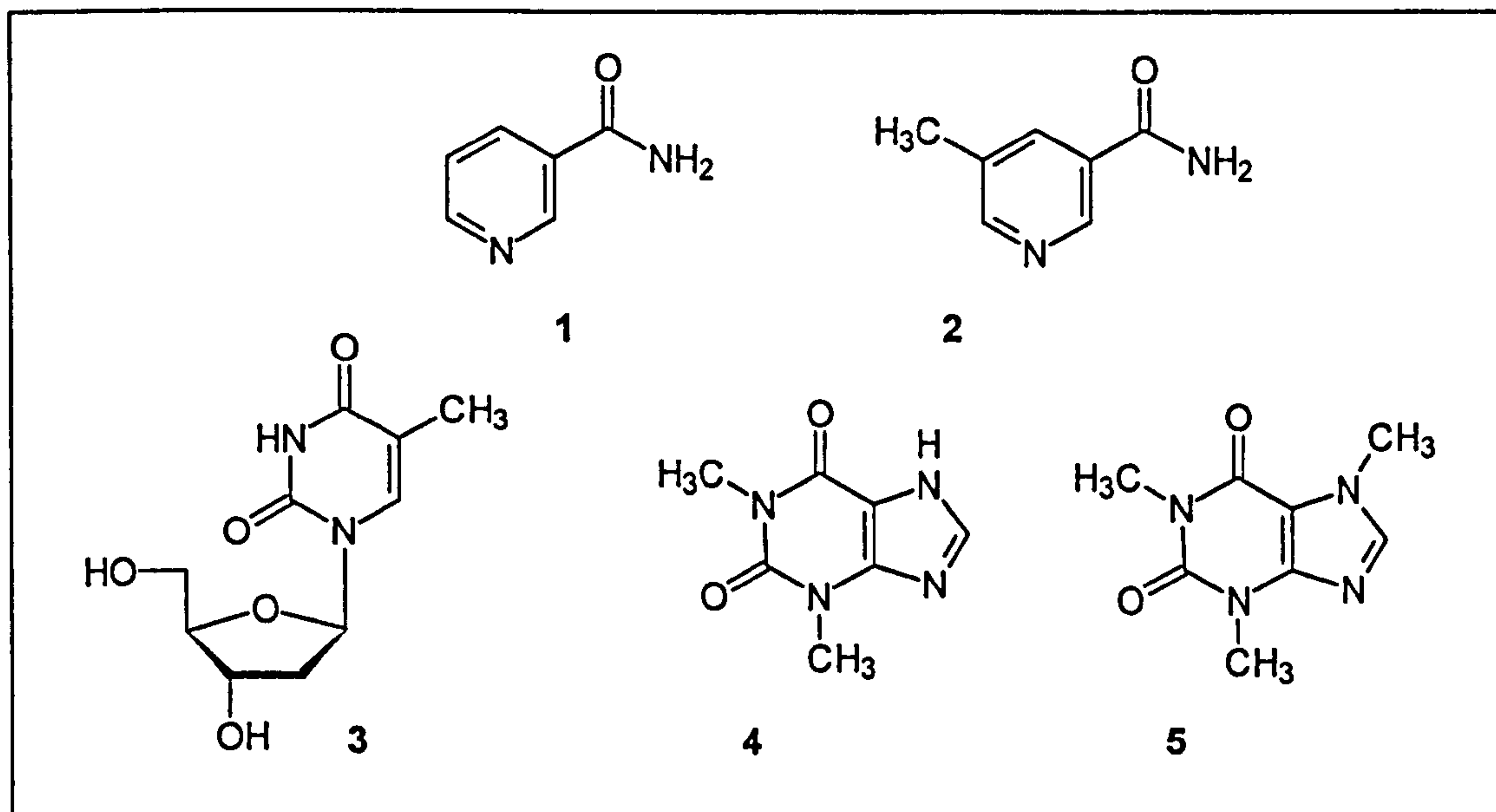
As the amount of knowledge concerning the role of the PARP enzyme continues to expand, so too does the search for specific, potent PARP inhibitors, with potential clinical value. Most of the information surrounding PARP has been gathered from experiments utilising PARP inhibitors as biological probes. The most immediate concern with chemical inhibitors, however, is the lack of potency and specificity exhibited towards PARP. Unfortunately, due to the presence of non-specific side effects with many of the inhibitors, the accurate interpretation of enzyme-inhibitor experiments is hampered, both with respect to the use of chemicals as biological probes and as clinical agents. Not only does this lead to misleading assumptions in the laboratory but the presence of non-specific side effects associated with many of the inhibitors would limit their effectiveness as clinical agents.

A large number of compounds are known to inhibit PARP to varying degrees *in vitro* or *in vivo*. Current attention appears to be directed towards the growing knowledge of structure-activity relationships, which are of crucial importance in designing and synthesising more potent and specific chemical inhibitors.

4.2 Development of PARP Inhibitors

The earliest inhibitors of PARP, identified as nicotinamide **1** and 5-methylnicotinamide **2**, were shown by Clark *et al* to function as competitive inhibitors of the enzyme.²²⁹ Other compounds used in early studies include thymidine **3**,^{230,231} theophylline **4**²³¹ and caffeine **5**.⁴⁹ It was quickly realised, however, that these compounds had activities on other biological processes in addition to the inhibition of PARP. Nicotinamide **1** also serves as a substrate for nicotinamide-*N*-methyltransferase deaminase and phosphoribosyl

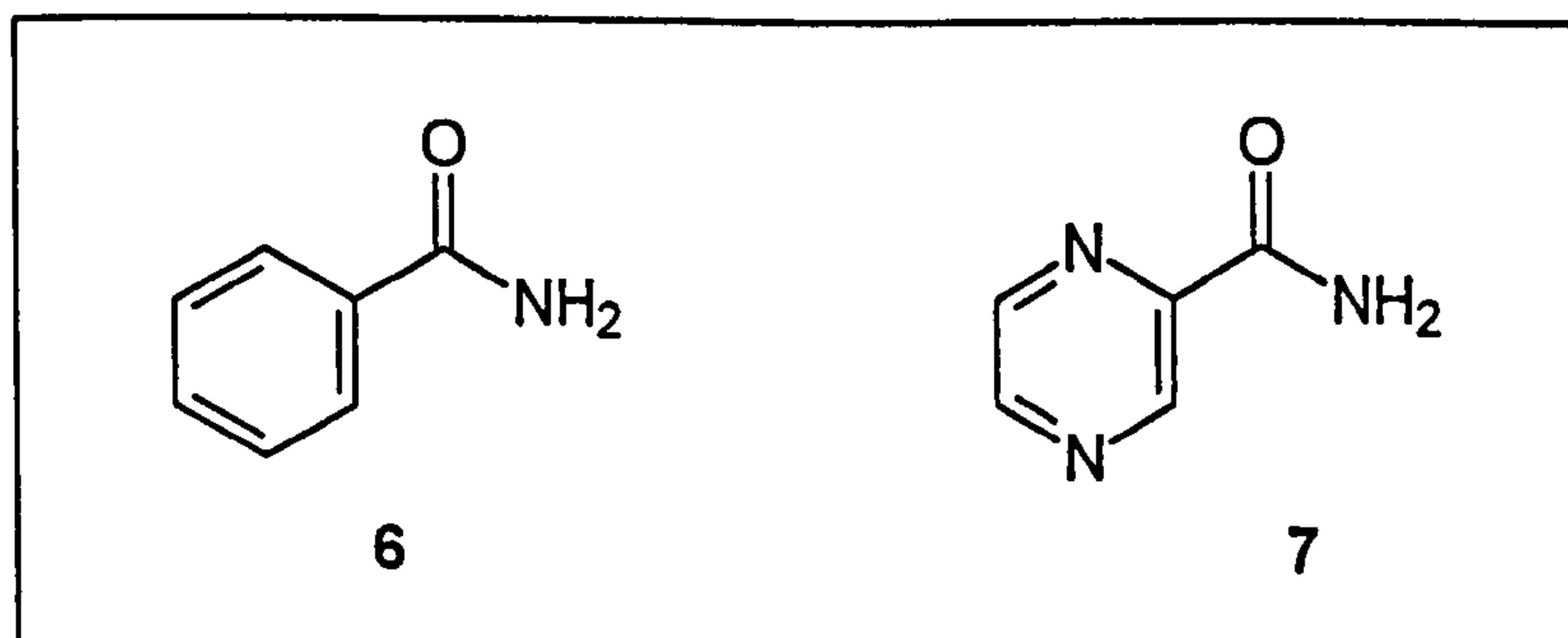
transferase.²³² It is an inhibitor of microsomal NADase, mono(ADP-ribosyl)transferases and adenosine 3', 5'-cyclic monophosphate phosphodiesterase.²³³



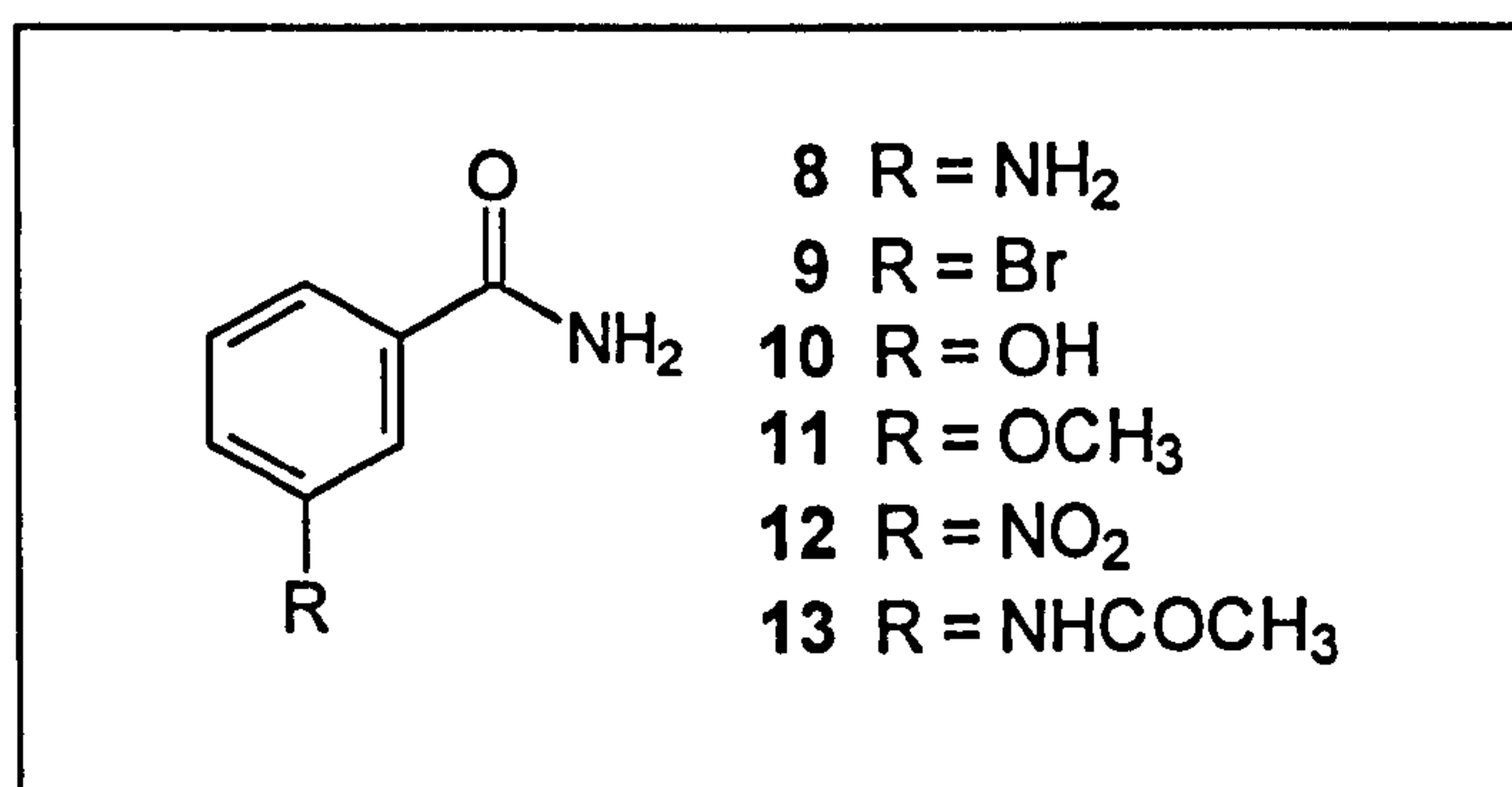
Thymidine 3 is converted to a phosphorylated species which subsequently inhibits the synthesis of 2'-deoxycytidine phosphate and results in cell division arrest.²³⁴ Theophylline 4 inhibits cyclic nucleotide phosphodiesterase²³⁵ and, along with caffeine 5, inhibits DNA repair.²³⁶ Therefore, the effects of compounds 1-5 on cellular metabolism cannot be solely attributed to the inhibition of PARP.

4.2.1 Nicotinamide and Benzamide Analogues

In 1975, Shall reported the efficacy of the two nicotinamide isosteres, benzamide 6 and pyrazinamide 7 as PARP inhibitors.²³⁷



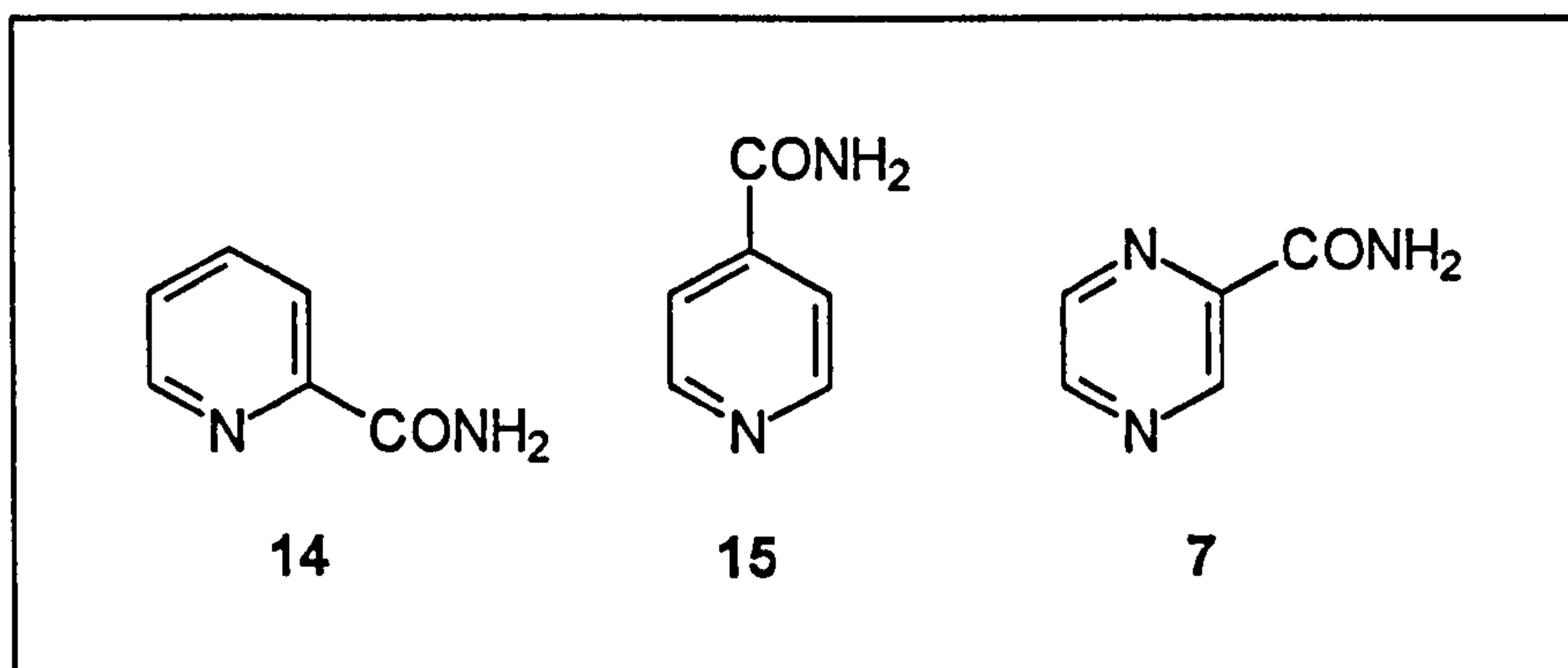
Purnell and Whish subsequently investigated the inhibitory activities of benzamide 6 and structurally related compounds, in an attempt to find more physiologically specific PARP inhibitors.²³⁸ Under their experimental conditions, benzamide was found to be a more potent inhibitor than nicotinamide and thymidine. A number of 3-substituted benzamides (8-13), were also tested by Purnell and Whish. The introduction of polar substituents onto the aromatic ring of benzamide served to increase the aqueous solubility of the compounds compared to benzamide alone, without compromising inhibitory activity.²³⁸ 3-Aminobenzamide (3AB) 8 and 3-methoxybenzamide 11 were shown to be competitive inhibitors and are generally regarded as 'benchmark' inhibitors of PARP.



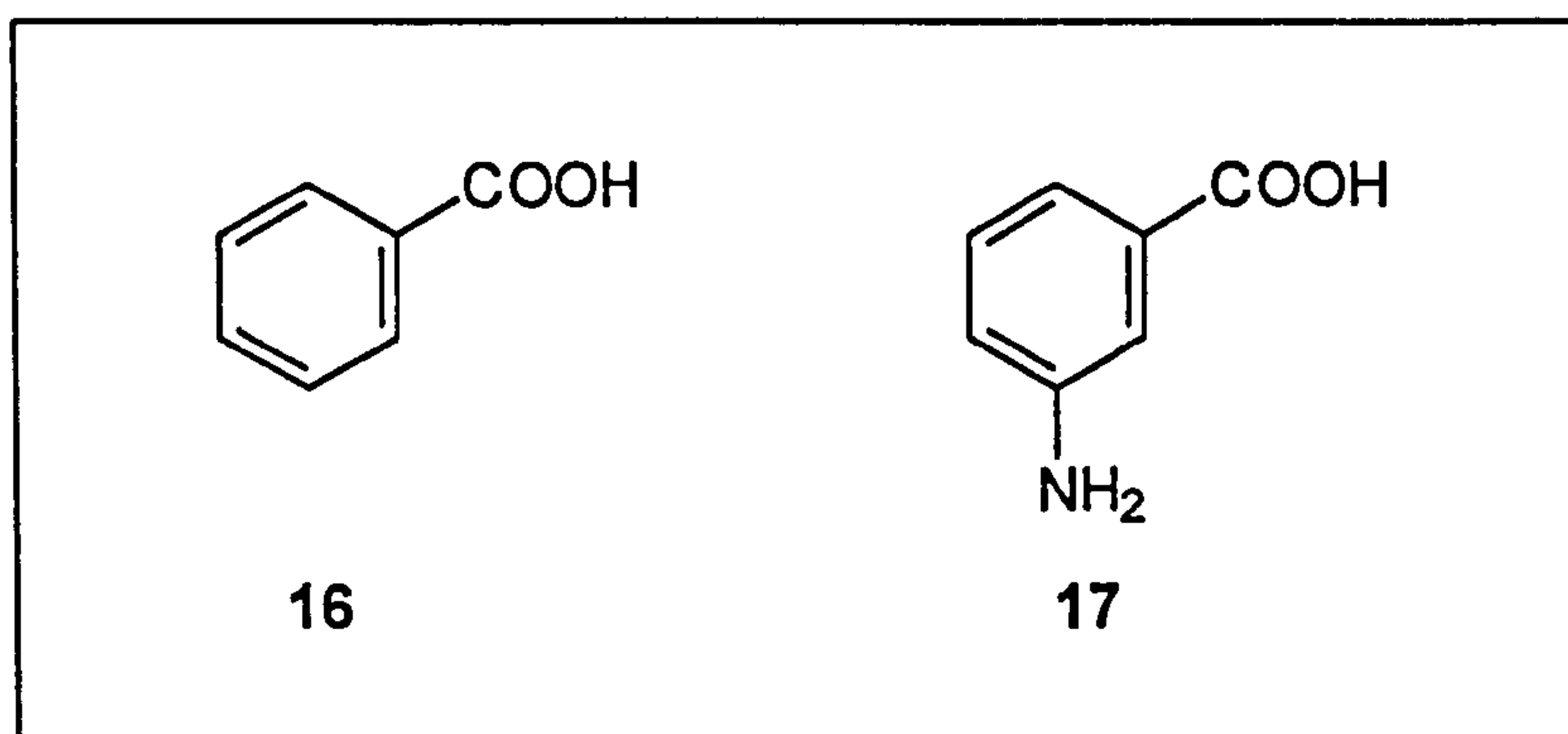
Modification of the carboxamide moiety was also investigated in the 3-substituted benzamides and the authors concluded that replacement of the carboxamide group with a carboxylate group greatly reduced or abolished enzyme-inhibitory activity, as did alkylation of the carboxamide substituent.²³⁸ Interestingly, the acylated analogue of 8, 3-acetamidobenzamide 13 retained potent inhibitory activity. Overall, the absence of a ring nitrogen in the benzamide analogues compared to the nicotinamide derivatives serves to abolish the metabolism of benzamide analogues by NAD⁺-metabolising enzymes.

Since the discovery of the potent inhibitory activity of the 3-substituted benzamides, a variety of detailed investigations have been undertaken by independent research groups. It is important to note that the many discrepancies reported in the literature on inhibitory activity values for given compounds, arise through the wide range of experimental cell systems, techniques and cytotoxic agents used. Therefore, direct comparison of biological results should be interpreted cautiously.

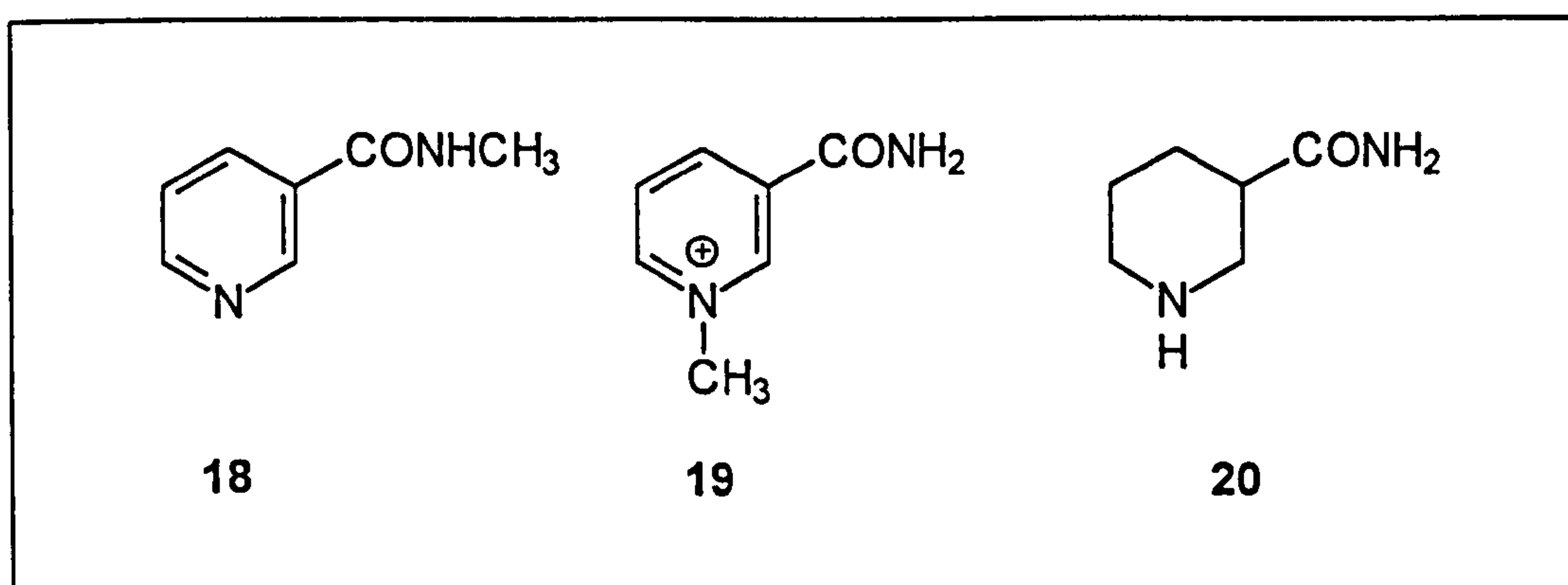
In terms of structure-activity relationships, the first systematic analysis of PARP inhibitors was conducted by Sims *et al.*¹⁹⁰ They tested thirty-three analogues of the nicotinamide and adenine portions of NAD⁺, using a permeabilised cell assay. Of the compounds tested, they found benzamide 6, 3AB 8 and 3-methoxybenzamide 11 to be the most potent PARP inhibitors, causing 96% inhibition of the enzyme under their experimental conditions.¹⁹⁰ Sims *et al* confirmed the earlier assumptions of Purnell and Whish regarding the absence of a ring nitrogen in the benzamide derivatives, and also demonstrated that a carboxamide group in the 3-position of the aromatic ring was optimal for enzyme inhibition. However, derivatives with a carboxamide group in the 2- or 4-position (picolinamide 14 and isonicotinamide 15) also exhibited reasonable inhibitory activity, although were less potent than nicotinamide itself.¹⁹⁰



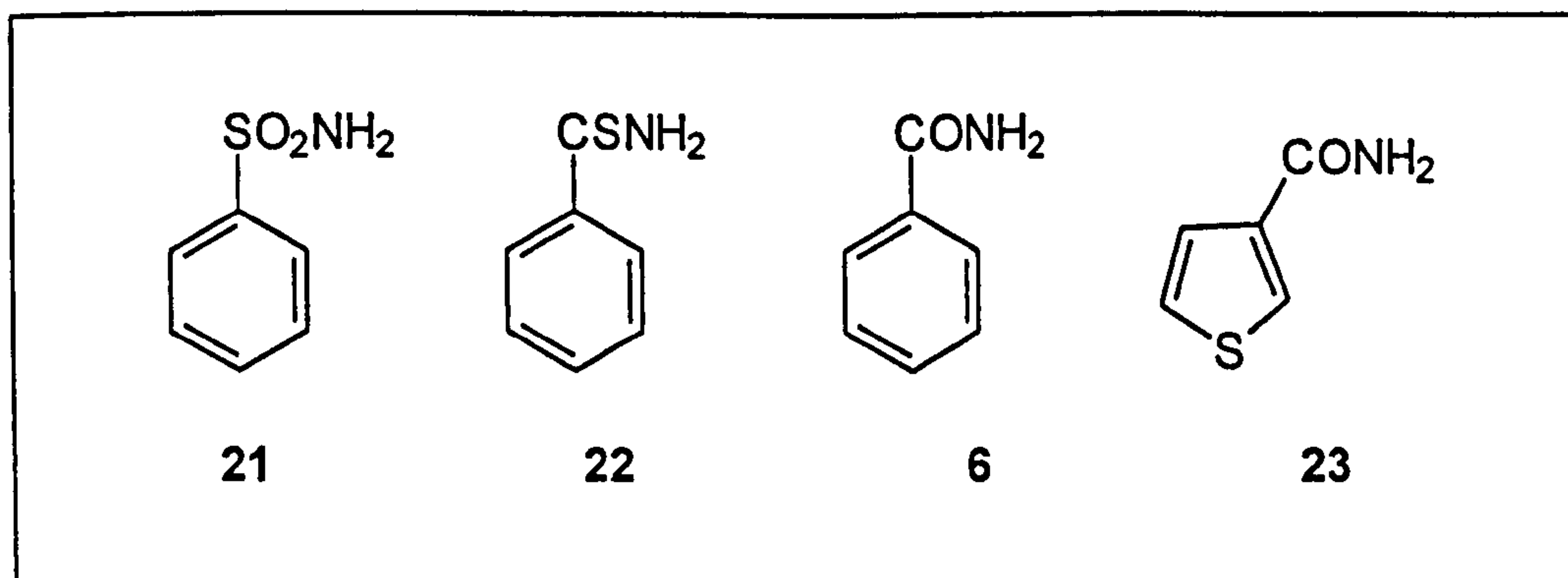
Pyrazinamide 7 was only slightly less potent than nicotinamide, indicating that the introduction of a second ring nitrogen did not interfere with inhibitory activity.¹⁹⁰ In agreement with Purnell and Whish,²³⁸ Sims *et al* also found that replacement of the carboxamide moiety with a carboxylate group resulted in the abolition of activity. For example, at a concentration of 2 mM for each compound, benzamide 6 and 3AB 8 showed 96% inhibition whereas benzoic acid 16 and 3-aminobenzoic acid 17 showed no inhibition.¹⁹⁰



It was determined that *N*-alkylation of the carboxamide (18) or the ring nitrogen (19), resulted in a substantial decrease of inhibitory activity, as did reduction of the aromatic ring conjugated to the carboxamide group (20).



Cantoni and coworkers investigated the inhibitory activity of sulphur containing benzamide derivatives in an attempt to probe further structure-activity requirements.²³⁹ They carefully selected compounds which had a gradual increase in dipole moments, hydrogen bonding strengths and steric hindrance of the amide functional group (sulfonamide > thioamide > carboxamide).



The sulphonamide **21** and thioamide **22** analogues were found to be much less active than benzamide **6**, as too was thiophene-3-carboxamide **23**, although this compound is regarded as a classical bioisostere of benzamide.²³⁹

4.2.2 Specificity of PARP Inhibitors

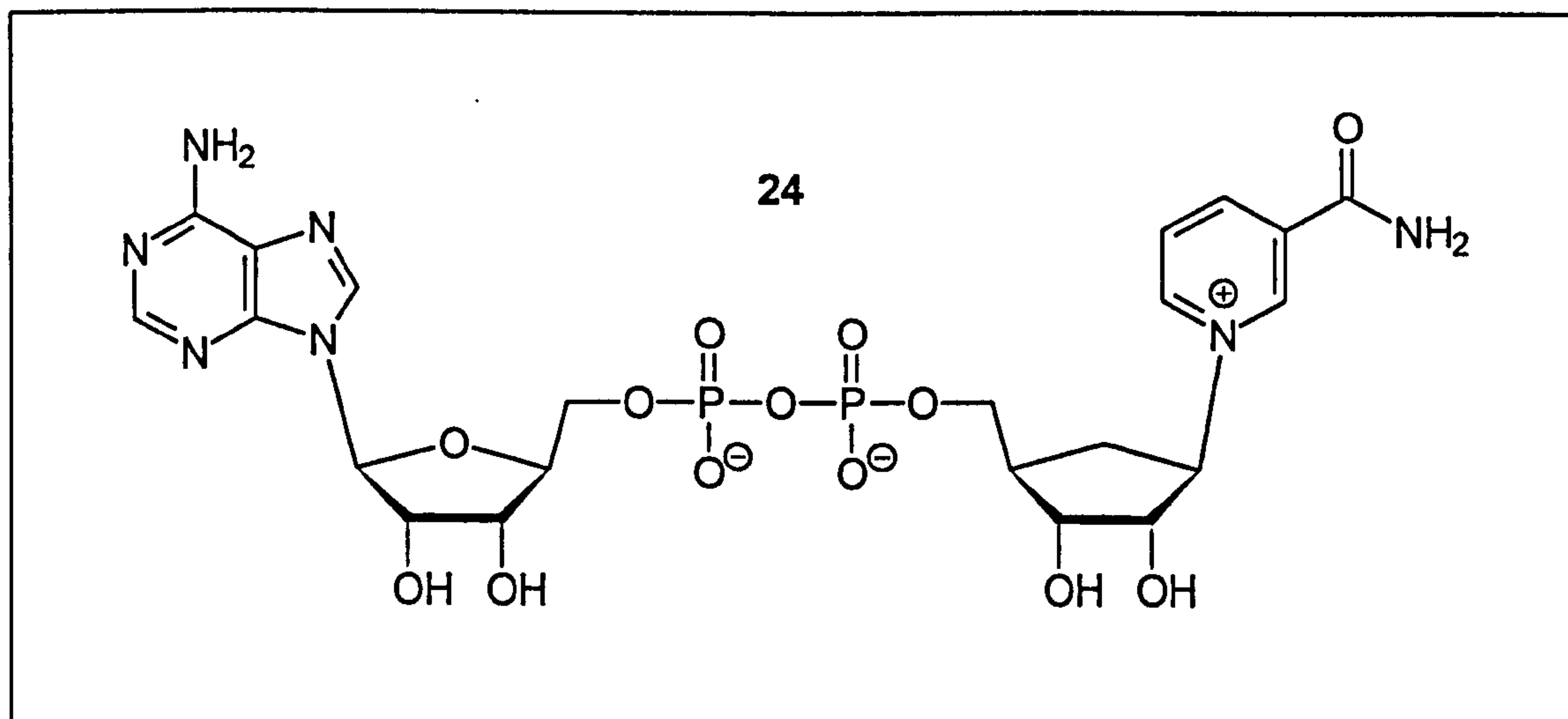
The specificity of the nicotinamide and benzamide PARP inhibitors has been questioned by various authors^{142,143} and important quantitative studies on PARP inhibitors were carried out by Rankin *et al.*²⁴⁰ Since ADP-ribosylation reactions are carried out by PARP, mono(ADP-ribosyl) transferases and NAD⁺ glycohydrolases, Rankin *et al* evaluated the selectivity of compounds, previously identified as PARP inhibitors, on all three classes of enzymes. The results indicated that at the micromolar concentrations of inhibitors necessary for the inhibition of PARP, the effects on mono(ADP-ribosyl) transferases and NAD⁺ glycohydrolases were minimal, suggesting that the inhibitors were indeed specific for PARP.²⁴⁰

However, in a subsequent investigation carried out by Sestili *et al*, it was demonstrated that the *in vitro* activities of inhibitors did not necessarily correlate with their *in vivo* activities.²⁴¹ In terms of structure-activity relationships, Sestili and coworkers confirmed earlier findings regarding structural requirements. In accordance with Purnell and Whish,²⁴¹ they agreed that acylation of the amino substituent of 3AB enhanced potency whereas alkylation made no apparent difference. They also concluded that no simple relationship between partition coefficients, dipole moments and biological activity existed for the benzamide analogues.²⁴¹

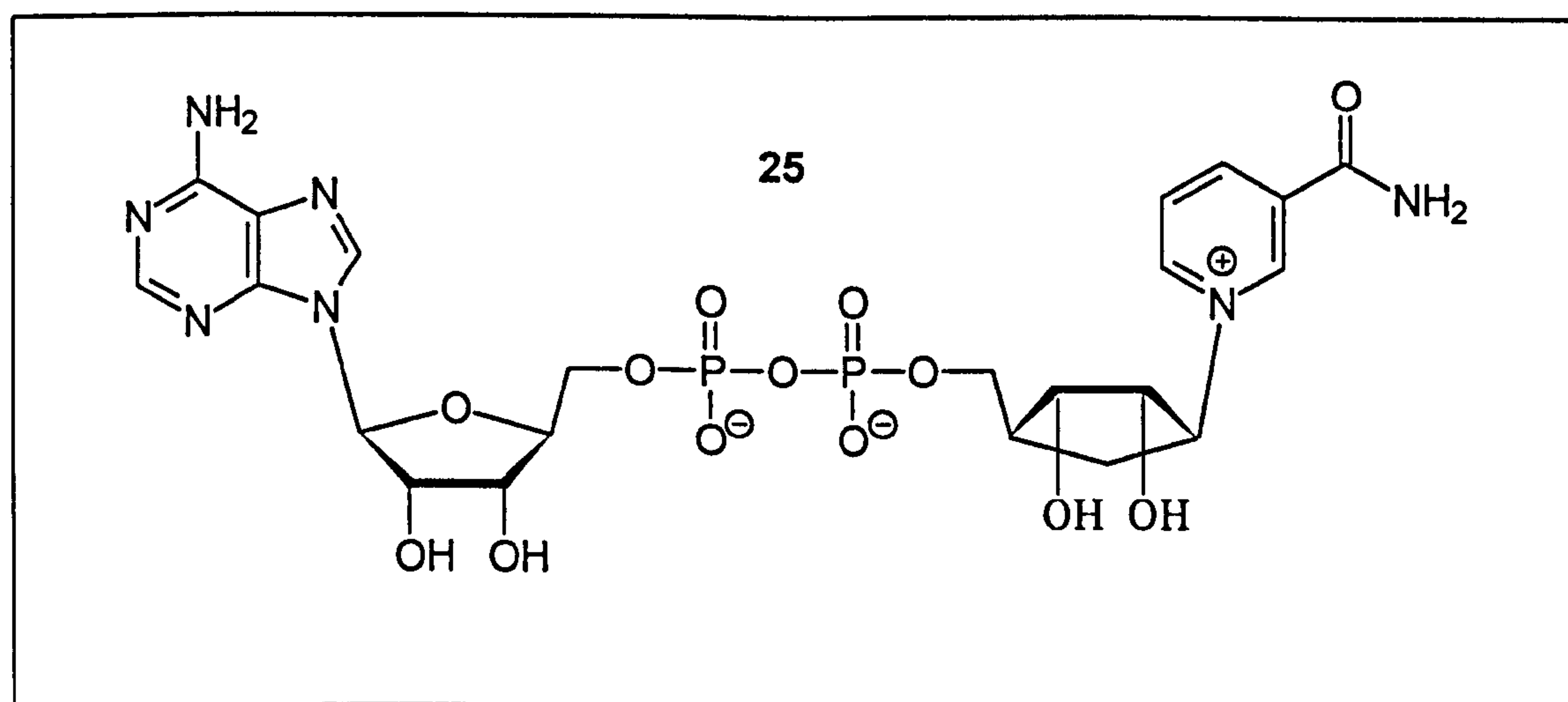
4.2.3 Mechanism-Based PARP Inhibitors

A series of compounds designed to interfere with the ADP-ribosylation reaction catalysed by PARP, was synthesised by Slama and Simmons.¹⁰¹ They replaced the β -D-ribosyl ring of the nicotinamide ribonucleoside moiety of NAD⁺ with a 2,3-dihydroxycyclopentane ring. The resulting compound, carba-NAD⁺ **24** was expected to

function as a mechanism-based inhibitor, as replacement of the oxygen atom would render the 'nicotinamide-ribose' bond resistant to cleavage by PARP. The derivative otherwise closely resembles NAD^+ in shape and overall charge.



Because of this resemblance, however, carba- NAD^+ has been shown to be an efficient substrate for alcohol dehydrogenases from yeast and horse liver.¹⁰¹ The diastereoisomer, ψ -carba- NAD^+ **25** has the incorrect geometry for binding and catalysis and is neither a substrate nor an inhibitor of either dehydrogenase.

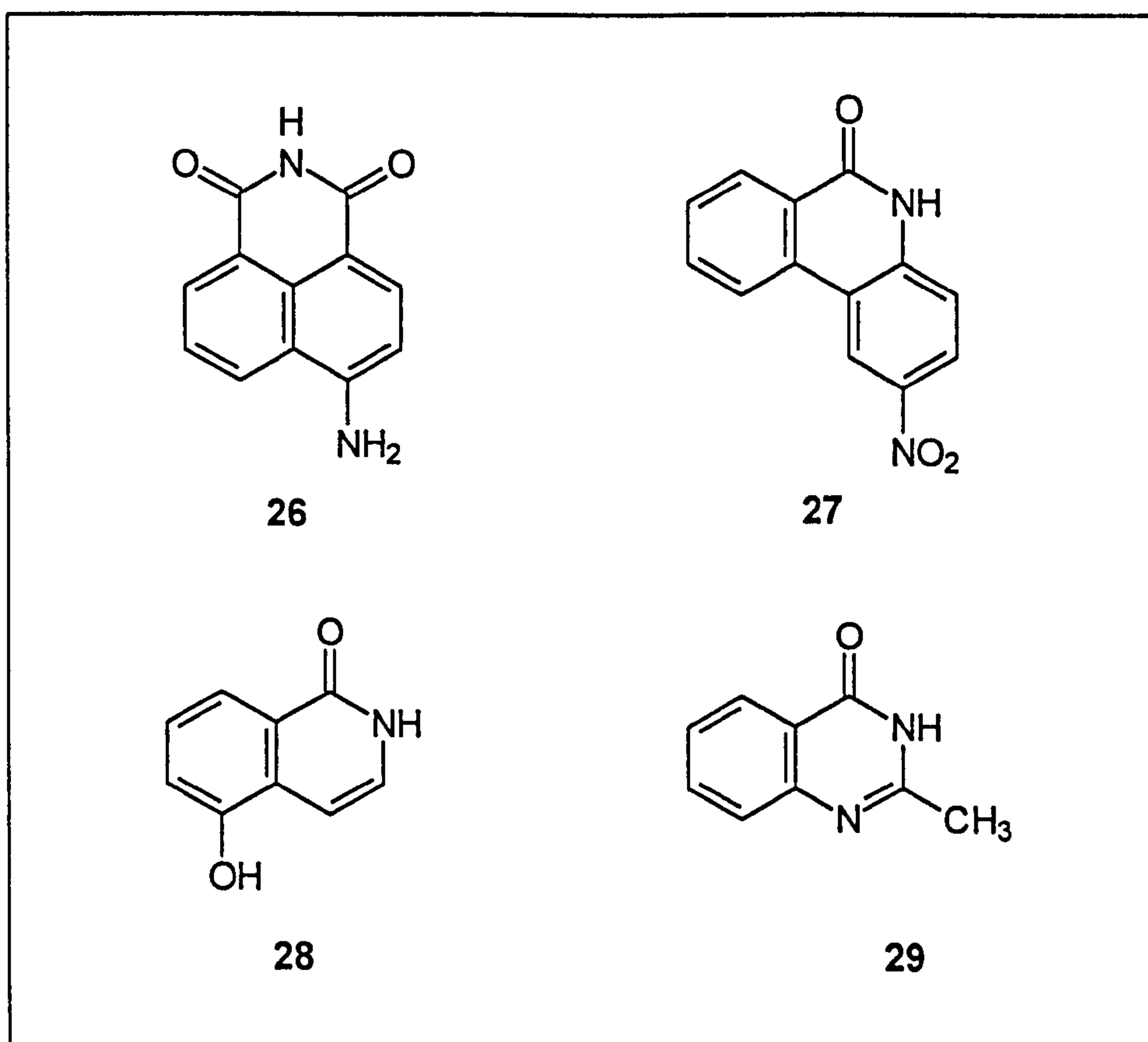


As a diastereoisomeric mixture, **24** and **25** were shown to be effective inhibitors of the mono(ADP-ribosyl) transferases. Surprisingly, when the mixture was separated, the

inhibitory activity was attributed to ψ -carba-NAD⁺ 25, the 'unnatural' diastereoisomer.¹⁰¹ The carba-NAD⁺ analogues were found to be poor PARP inhibitors and would be limited in their effectiveness, because of their abilities to interfere with other NAD⁺-metabolising enzymes.

4.2.4 Conformationally Restricted Inhibitors of PARP

Banasik *et al* identified an interesting class of PARP inhibitors possessing a unifying structural feature: the carboxamide group conjugated to the aromatic ring was constrained within a ring system.^{242,243} Compounds evaluated included 4-amino-1,8-naphthalimide 26, 2-nitro-6-[5H]-phenanthridinone 27, 1,5-dihydroxyisoquinoline 28 and 2-methylquinazolin-4-[3H]-one 29.

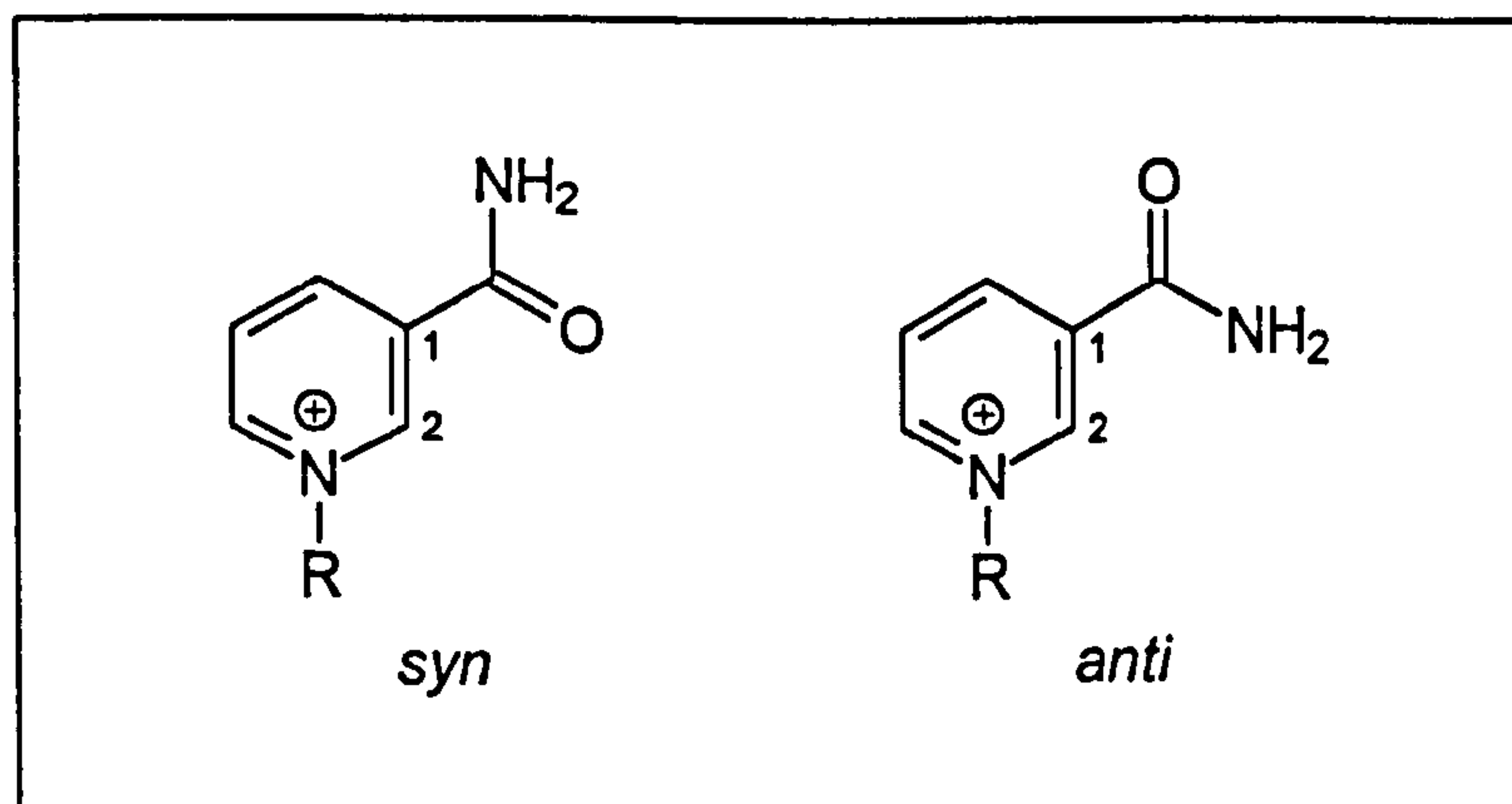


Compound 26 proved over 100-fold more potent against PARP than benzamide or 3AB and was by far one of the most active inhibitors evaluated at the time.^{242,243} As with

previous studies, Banasik *et al* found that the majority of these very potent inhibitors were selective for PARP over the mono(ADP-ribosyl) transferases. Molecular orbital calculations were performed to determine which part of the inhibitors contributed most effectively towards inhibitory activity.²⁴² The results indicated that a controlling factor for inhibitory activity was the ability of the oxygen atom of the carboxamide group to donate π -electrons to a positively charged region within the enzyme active-site. Thus, Banasik *et al* suggested that the oxygen atom served as an electron donor to a putative hydrogen-bond acceptor within the catalytic domain.²⁴² Since the activity of inhibitors bearing *N*-substituted carboxamides was poor, it was also inferred that an unsubstituted carboxamide group is required to act as an electron acceptor to a putative hydrogen-bond donor.

Further information regarding the conformation of the carboxamide group of NAD^+ in the enzyme active-site was gathered from the computational work of Li and Goldstein.²⁴⁴ Their results, based on *ab initio* calculations of the nicotinamide ring of NAD^+ , indicated that the carboxamide group adopts a specific orientation relative to the enzymic catalytic groups within the active-site. Two major orientations of the carboxamide group were proposed, as depicted in *Figure 4.2.4.1*.

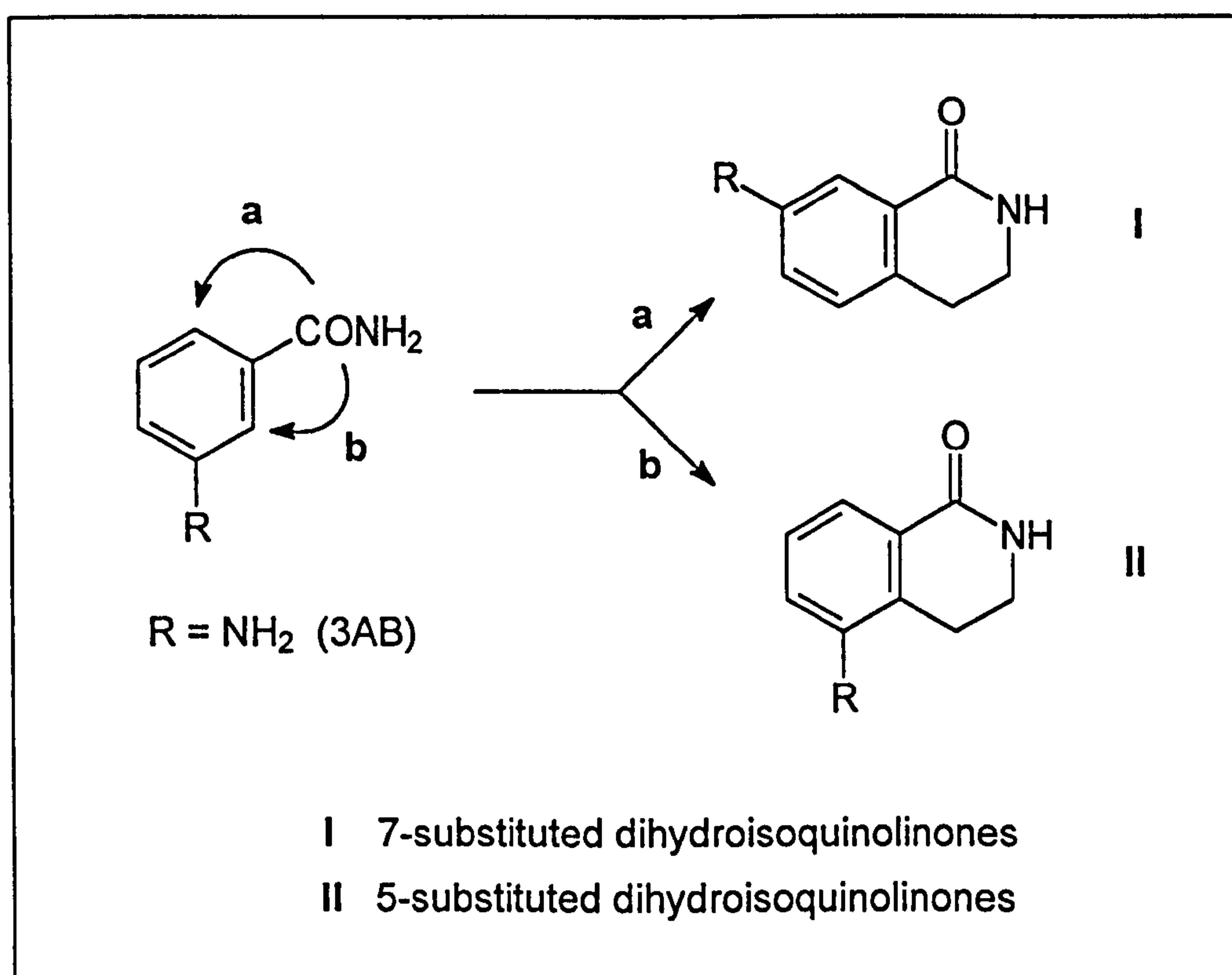
Figure 4.2.4.1 Possible orientations of the carboxamide group of NAD^+



Li and Goldstein's results demonstrated that the biologically active conformation was that with the carbonyl group orientated *anti* to the 1,2-bond of the aromatic ring.

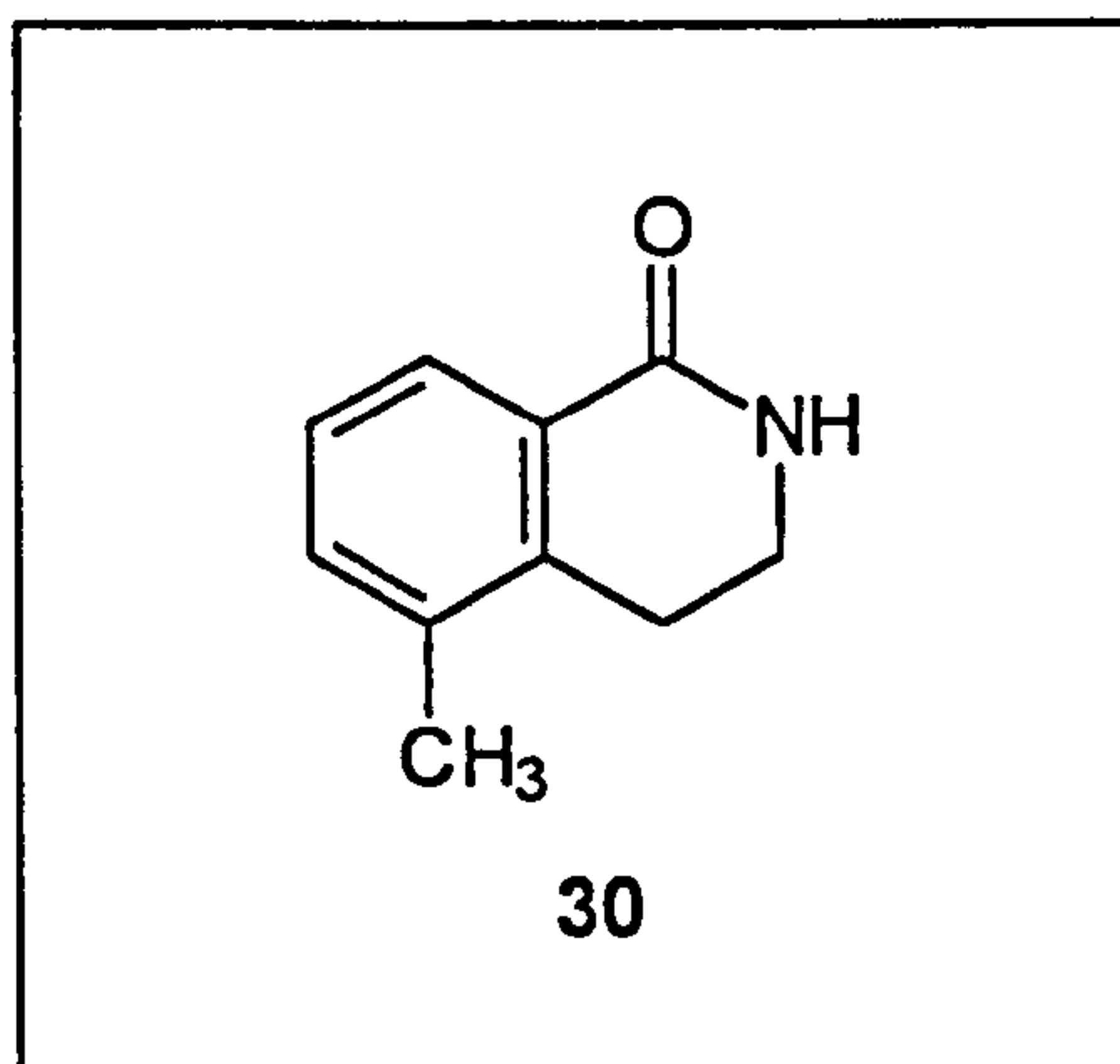
Evidence in support of these findings was provided by Suto *et al*, who independently investigated conformationally restricted compounds.²⁴⁵ Using the 3-substituted benzamides as a template, they synthesised compounds containing an ethane bridge linking the benzene ring to the amide nitrogen (see *Figure 4.2.4.2*).²⁴⁵

Figure 4.2.4.2 Design of conformationally restricted benzamide analogues²⁴⁵



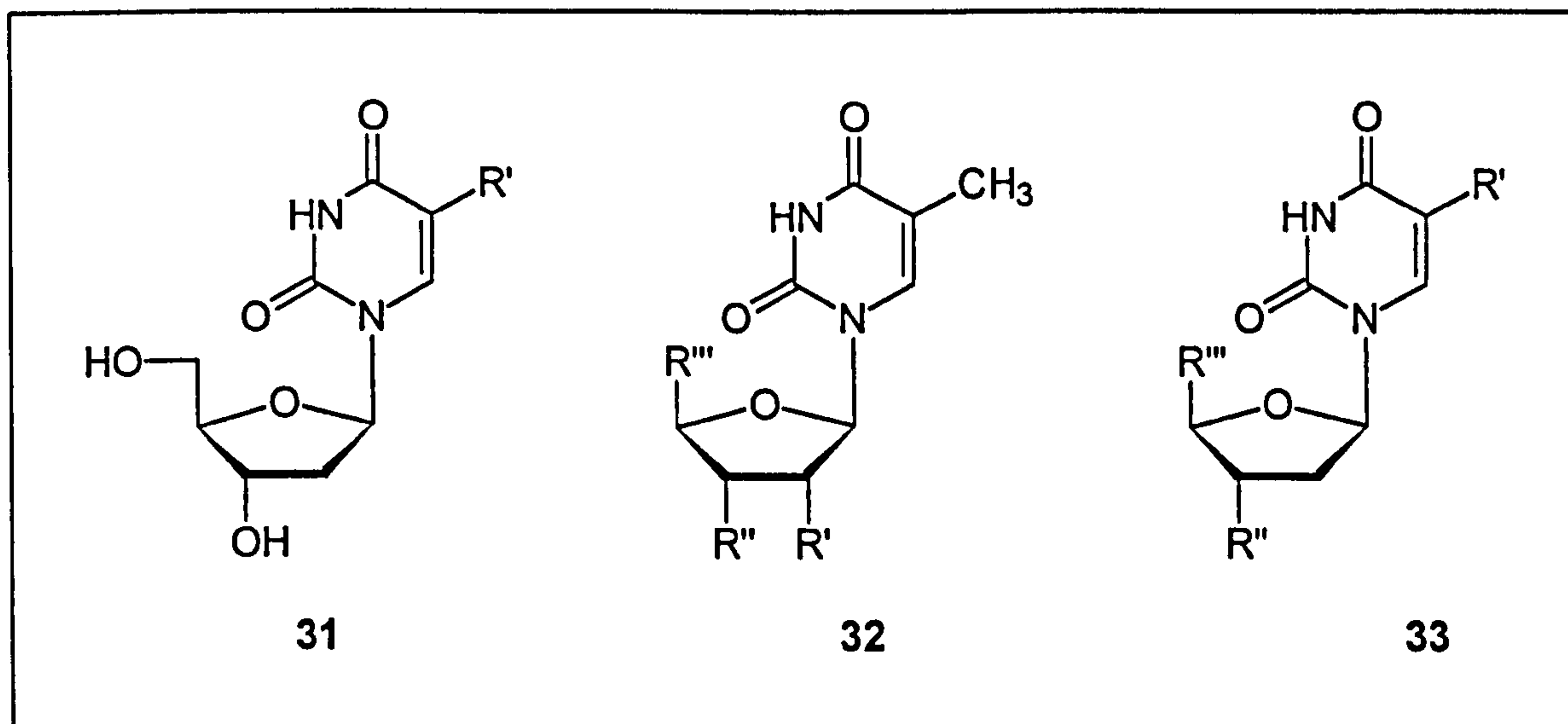
It was found that the 5-substituted dihydroisoquinolinones II, were extremely potent inhibitors of PARP, and were 50-75 fold more potent inhibitors of the polymerase than the 7-substituted dihydroisoquinolinones I. Suto *et al* concluded that the positioning of the aromatic substituent, along with restriction of the carboxamide rotation into the *anti* configuration, was critical for potent activity, as a decrease in activity was observed when the ring substituent was placed in the 6-, 7- or 8-position.²⁴⁵ The most potent inhibitor identified in the dihydroisoquinolinone series was PD128763 (3,4-dihydro-5-methyl-1-[2*H*]-isoquinolinone) 30 which, when tested against calf thymus PARP, proved to be 50 times more potent than 3AB 8 under the same conditions.²⁴⁶ It was suggested that owing

to its excellent PARP inhibitory activity, compound **30** may act as a radiosensitizer and thus be of clinical value for use in conjunction with radiotherapy.²⁴⁶



4.2.5 Nucleoside Analogues of Thymidine as PARP Inhibitors

A series of deoxyuridine and deoxythymidine analogues was evaluated for inhibitory activity against the PARP enzyme.²⁴⁷ Three classes of compounds were examined, as shown below (**31-33**).

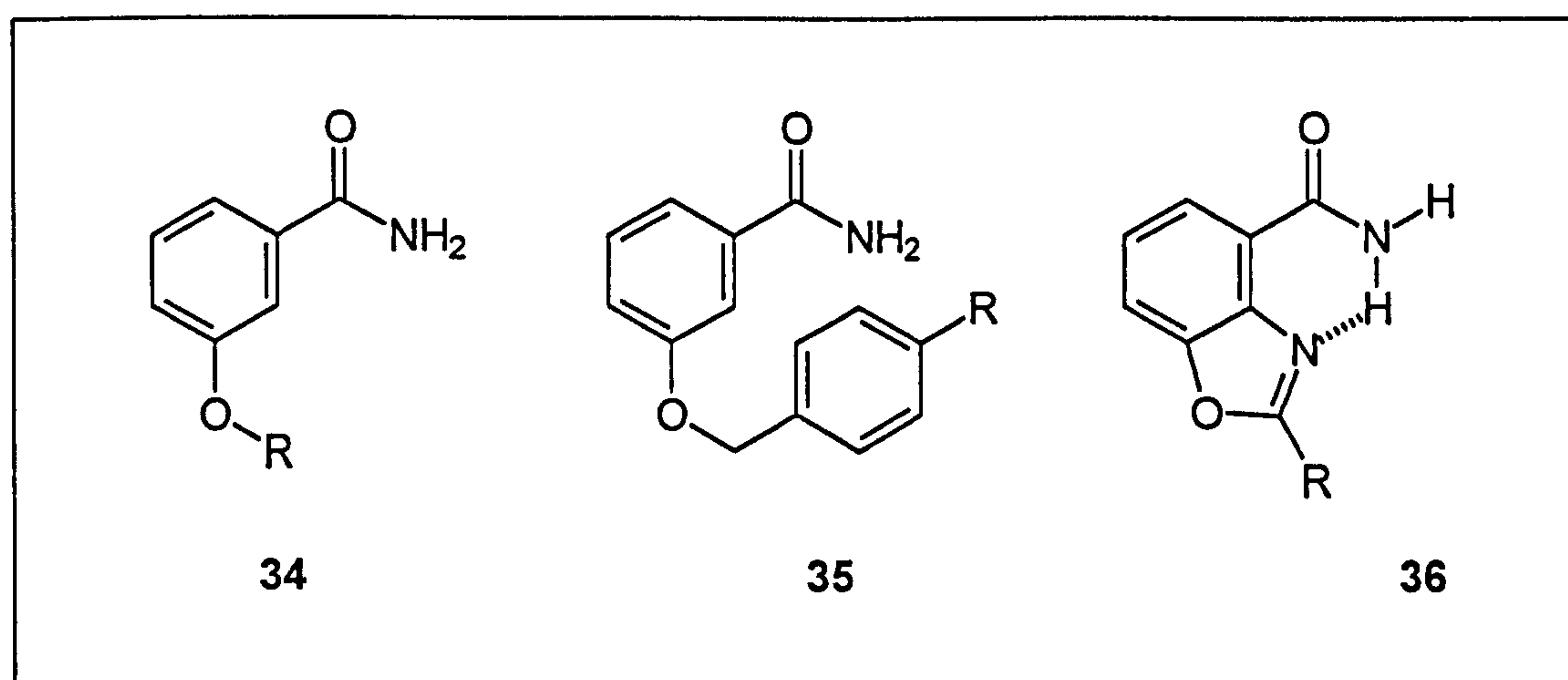


Under the experimental conditions used, various deoxyuridine and deoxythymidine derivatives were found to be potent inhibitors, with inhibition constant values (K_i) five-

fold lower than the corresponding value for 3AB. Interestingly, the same compounds are also known to exhibit antiviral and/or anticancer activities.²⁴⁷

4.2.6 Design and Synthesis of Heterocyclic PARP Inhibitors

As part of the Anticancer Drug Discovery Initiative (ADDI) programme at Newcastle University, a number of novel heterocyclic PARP inhibitors were developed. Using 3-hydroxybenzamide **10** as a template, a series of *O*-alkylbenzamide **34** and substituted benzyloxybenzamides **35** was synthesised.²⁴⁸ These compounds were designed to explore the ribose nucleotide-binding domain of PARP, and the steric, electronic and hydrogen-bonding properties of the compounds were widely varied.^{248,249}



From the variety of substituents introduced at the three-position of the benzamides, the authors concluded that the ribose nucleotide-binding domain appears to accommodate relatively bulky groups. The activities of the compounds as PARP inhibitors were described as unexceptional and no clear structure-activity requirements were gained from these analogues.²⁴⁹ A second novel approach was used for restricting the carboxamide moiety into the *anti* position. The benzoxazole-4-carboxamides **36** are electron-rich heterocycles and, as such, should enhance the hydrogen-bond donor properties of the carbonyl oxygen. An intramolecular hydrogen bond between the amide and oxazole nitrogens constrains the carboxamide group into the necessary conformation.²⁴⁹

The benzoxazole-4-carboxamides **36** were found to be more potent as PARP inhibitors than the benzyloxybenzamides **35**.²⁴⁹ As was observed previously, replacement of the carboxamide group of 2-methyl benzoxazole-4-carboxamide to give the corresponding carboxylate, resulted in loss of inhibitory activity, confirming the necessity of the amide functionally. *N*-methylation of the carboxamide group also resulted in a loss of activity, which indicating the importance of an unsubstituted amide group.²⁴⁹

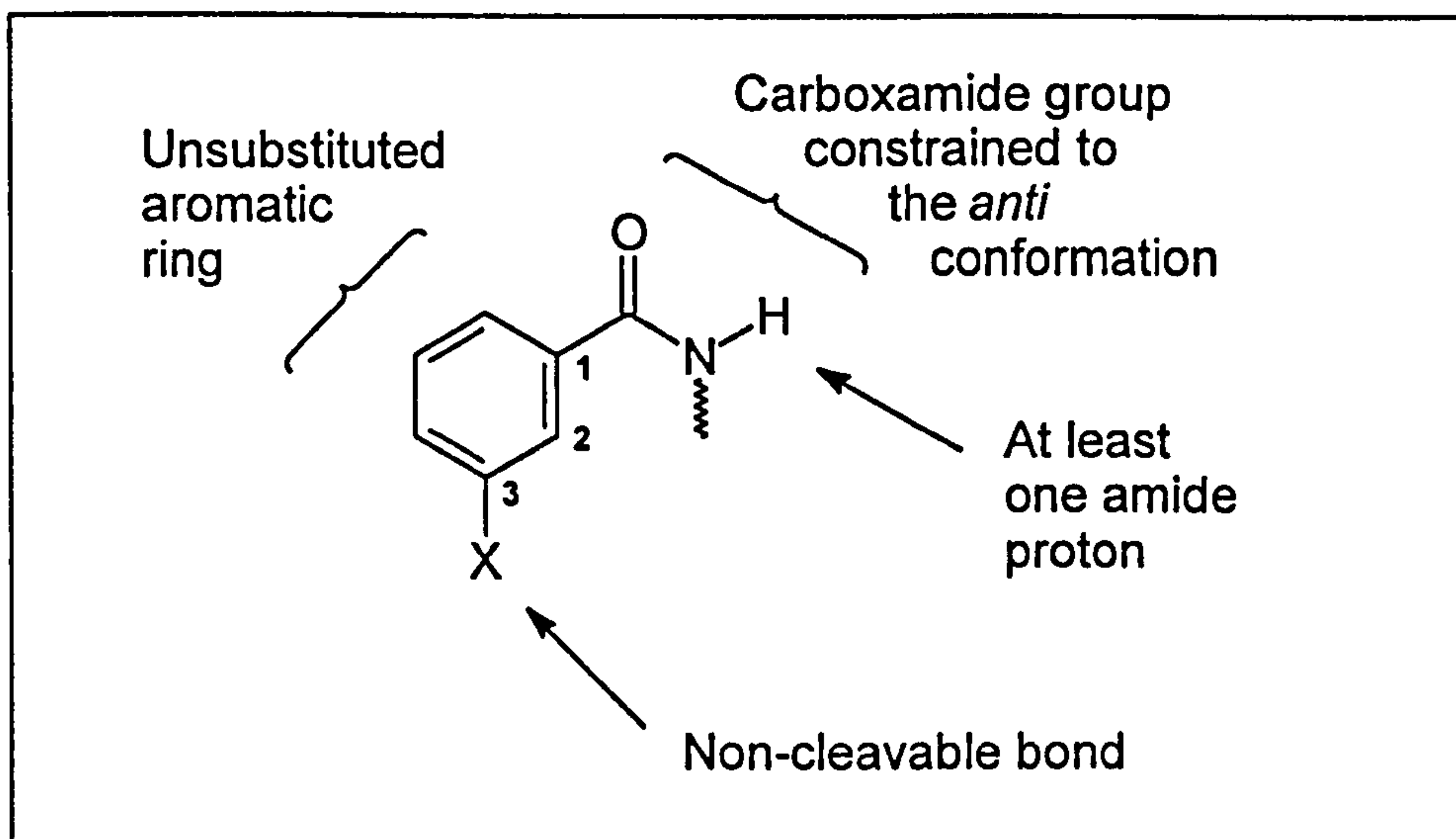
4.3 Summary of Structure-Activity Requirements

Thus far, it has been established that analogues of the natural substrate, NAD⁺, are effective inhibitors of PARP. Potent enzyme-inhibitory activity appears to be associated with the following features:

- i) an unsubstituted aromatic or polyaromatic heterocyclic system
(saturated analogues show poor activity)
- ii) the presence of a carboxamide group
(carboxylate and thiocarboxylate analogues have no activity)
- iii) restriction of the carboxamide moiety into the *anti* conformation
(derivatives with unrestricted carboxamides are less active)
- iv) the presence of at least one amide proton for putative hydrogen bonding
(*N*-alkylation of the carboxamide moiety results in loss of activity)
- v) a non-cleavable bond at a position corresponding to the 3-position of the benzamides
(serves to reduce the enzymic metabolism of the inhibitor)

These features are summarised in *Figure 4.3*.

Figure 4.3 Summary of the structure-activity requirements for potent PARP inhibition



With regard to the design of novel inhibitors, other factors must also be taken into consideration. Ideally, the inhibitor should possess functional sites which allow for elaboration of the molecule, in order to probe the enzyme-active site. If the inhibitor is to be of clinical value as a therapeutic agent, then solubility problems must be addressed and a water-solubilising group may be necessary.

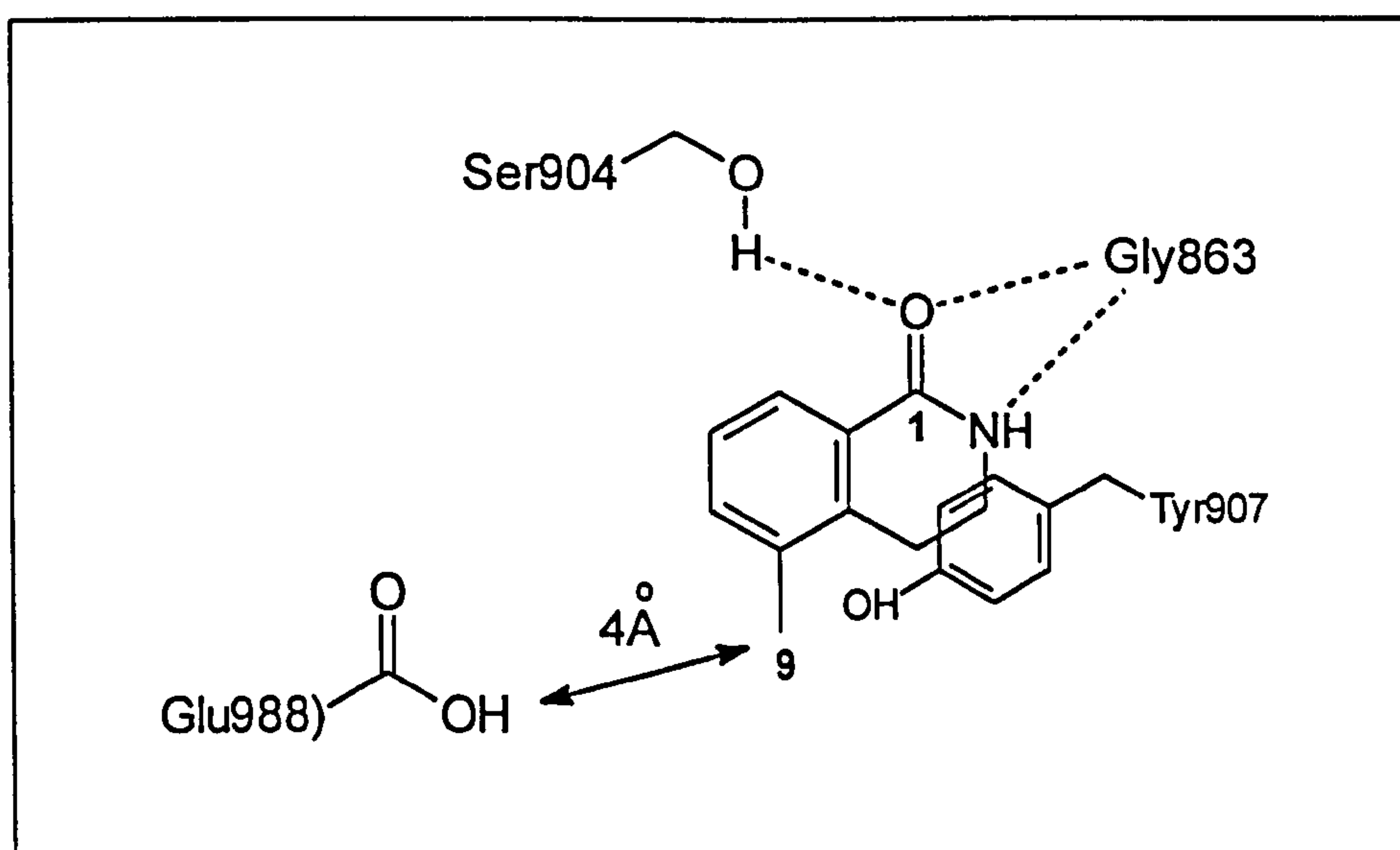
4.4 The Structure of the Enzyme-Active Site

The recently solved crystal structure of the catalytic fragment of chicken PARP has provided conclusive information with regard to enzyme-inhibitor binding.²⁵⁰ Since the catalytic domains of human and chicken PARP share 100% homology in amino-acid sequence, the information gained from this crystal structure is of potential value in the design of novel PARP inhibitors. Interestingly, the catalytic fragment of chicken PARP (PARP-CF) was found to be similar in structure to the catalytic domains of microbial toxins although the amino-acid sequence homology is relatively low.²⁵⁰

PARP-CF was crystallised with and without a nicotinamide-analogue inhibitor bound within the active-site.²⁵⁰ The inhibitor chosen was PD128763 **30**, the dihydroisoquinolinone derivative. The inhibitor was shown to be bound to PARP-CF by two hydrogen bonds from the carboxamide group to the peptide backbone of Gly863, and a third hydrogen bond from the carbonyl oxygen to the side chain of Ser904.²⁵⁰ It was assumed that the inhibitor binds to PARP-CF in an analogous manner to the nicotinamide portion of NAD⁺. Non-polar interactions between PD128763 and the adjacent Tyr907 were also identified.²⁵⁰

As mentioned in Section 2.5, Glu988 has been identified as a residue involved in catalysis.¹¹⁰ This residue is located at a distance of only 4Å from the methyl carbon atom (C9) of PD128763. It is thought that at this distance, the glutamate may act either as a general base to activate the substrate for nucleophilic attack, or to stabilise the intermediate oxocarbenium ion, formed during the course of the normal reaction. *Figure 4.4* shows a schematic representation of the enzyme-inhibitor interactions between PARP-CF and PD128763.²⁵¹

Figure 4.4 Enzyme-inhibitor interactions between PARP-CF and PD128763.²⁵¹



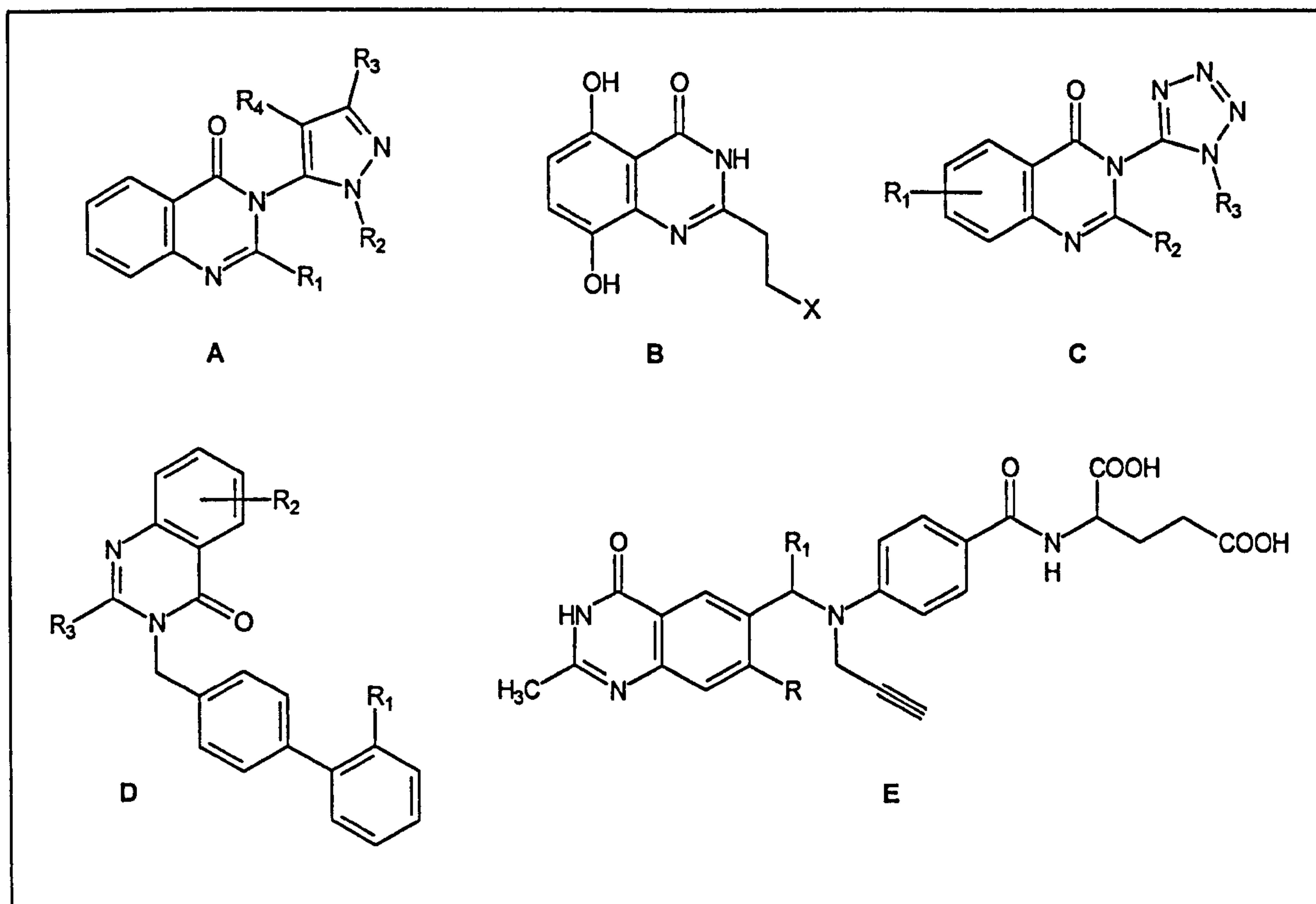
This crucial information has undoubtedly confirmed the earlier predictions regarding the structure-activity requirements of PARP inhibitors. These encouraging results indicate that the conformationally restricted inhibitors may bind in a manner analogous to PD128763 and the crystal structure can now be carefully examined and used to maximise enzyme-inhibitor interactions when designing new compounds.

Chapter Five: Development of the Quinazolinone Series

5.1 Quinazolinones as Pharmaceutical Agents

Certain compounds containing a quinazolinone moiety exhibit biological activities towards a variety of conditions. Many of these compounds have been developed as pharmaceutical agents, and *Figure 5.1* illustrates some examples of clinically useful quinazolinone derivatives.

Figure 5.1 Examples of Biologically Active Quinazolinones



The compounds of general structure A are 3-(5'-pyrazoyl)-3,4-dihydroquinazolin-4-ones and have been found to exhibit analgesic activity.²⁵² Compounds of structure B have been shown to be purine nucleoside phosphorylase inhibitors, which are used in the treatment of cancer, gout, malaria and organ rejection.²⁵³

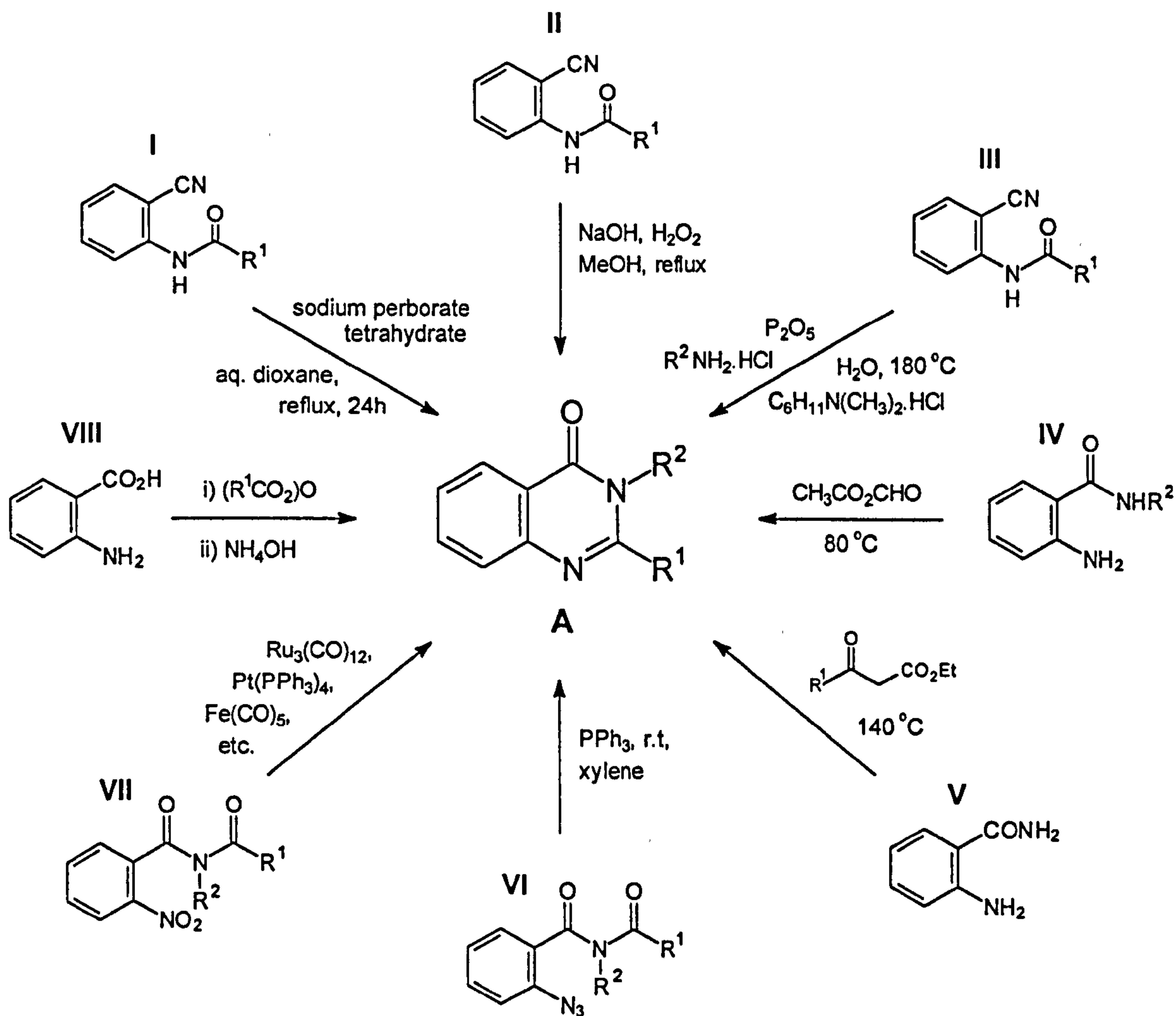
Compounds of structures **C**, **D**, and **E** have antiallergic, antihypertensive and antifolate properties, respectively.²⁵⁴⁻²⁵⁶

5.2 General Methods of Quinazolinone Synthesis

There are a number of methods available for the preparation of quinazolinone derivatives. *Scheme 5.2* illustrates some of the many ways that quinazolinone formation can be achieved [for methods **I**, **II**, **III**, **V** and **VIII**, $R^2 = H$]. It is well established that the quinazolinone skeleton can be readily formed from anthranilamide or anthranilic acid derivatives. In most cases, the reaction involves an intramolecular cyclisation (e.g. **I**, **II**, **III**, **IV**, **V** and **VIII**).

Method **I** is an example of the Radziszewski reaction whereby the oxidation of suitably functionalised *o*-amidobenzonitriles by sodium perborate leads to the facile cyclisation and formation of quinazolin-4-[3*H*]-ones.²⁵⁷ Compounds synthesised by methods **II** and **VIII** are intermediates in the synthesis of anti-hypertensive agents (structure **D**, *Figure 5.1*).^{255,258} Method **III** has been used for the preparation of the hypnotic drugs meclaqualone (*Scheme 5.2*, **A**: $R^1 = CH_3$, $R^2 = 2-Cl-C_6H_4$) and methaqualone (**A**: $R^1 = CH_3$, $R^2 = 2-CH_3-C_6H_4$)²⁵⁹ and quinazolinones obtained from method **IV** have proved to be of use as antiallergic agents.²⁵⁴ Method **VI** represents a mild, efficient synthesis involving an intramolecular aza-Wittig reaction²⁶⁰ and method **VII** is one of the few examples of quinazolinone formation by transition-metal catalysed reductive *N*-heterocyclisation.²⁶¹

Scheme 5.2 General methods of quinazolinone synthesis



5.3 Quinazolinones as PARP Inhibitors

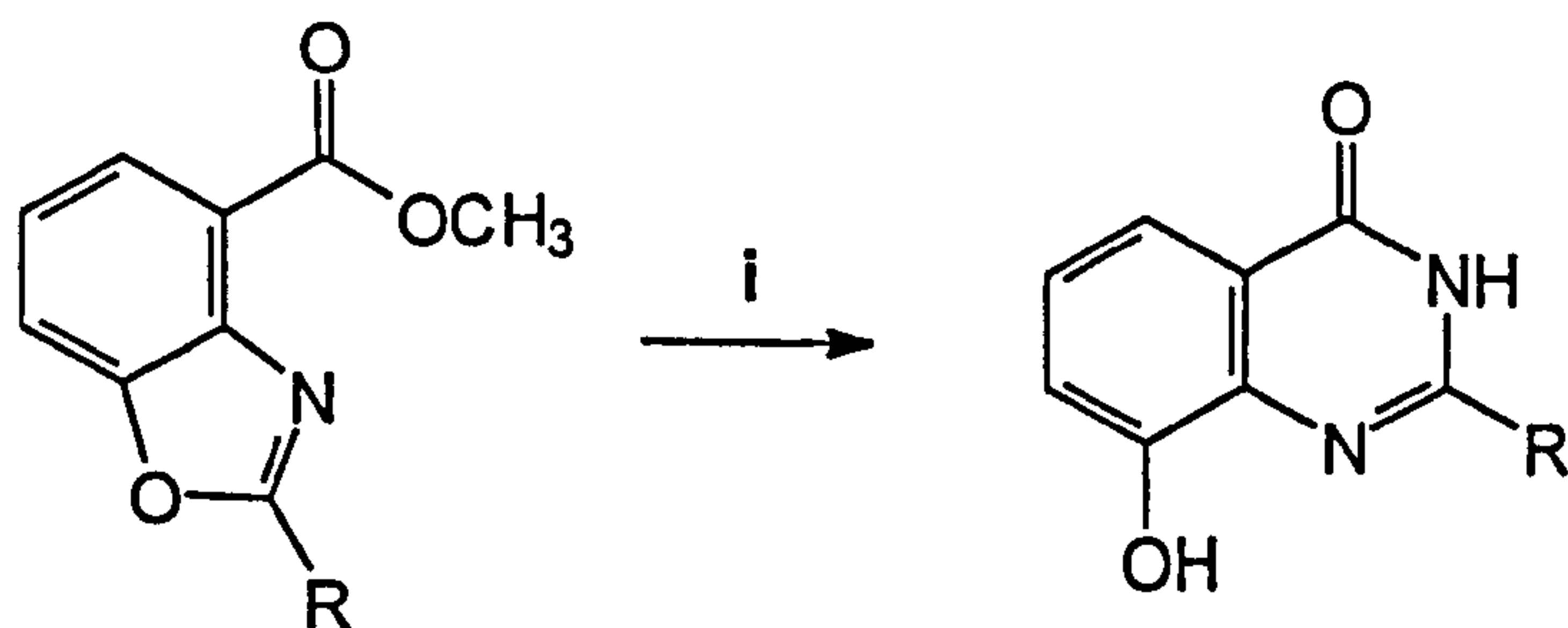
Quinazolinone derivatives have been largely neglected with respect to their inhibitory activity towards PARP, despite reports of 2-methyl quinazolin-4-[3H]-one exhibiting potent activity against the enzyme.^{232,249,262}

5.3.1 Rearrangement of Benzoxazole-4-carboxamides to 8-Hydroxyquinazolin-4-[3H]-ones

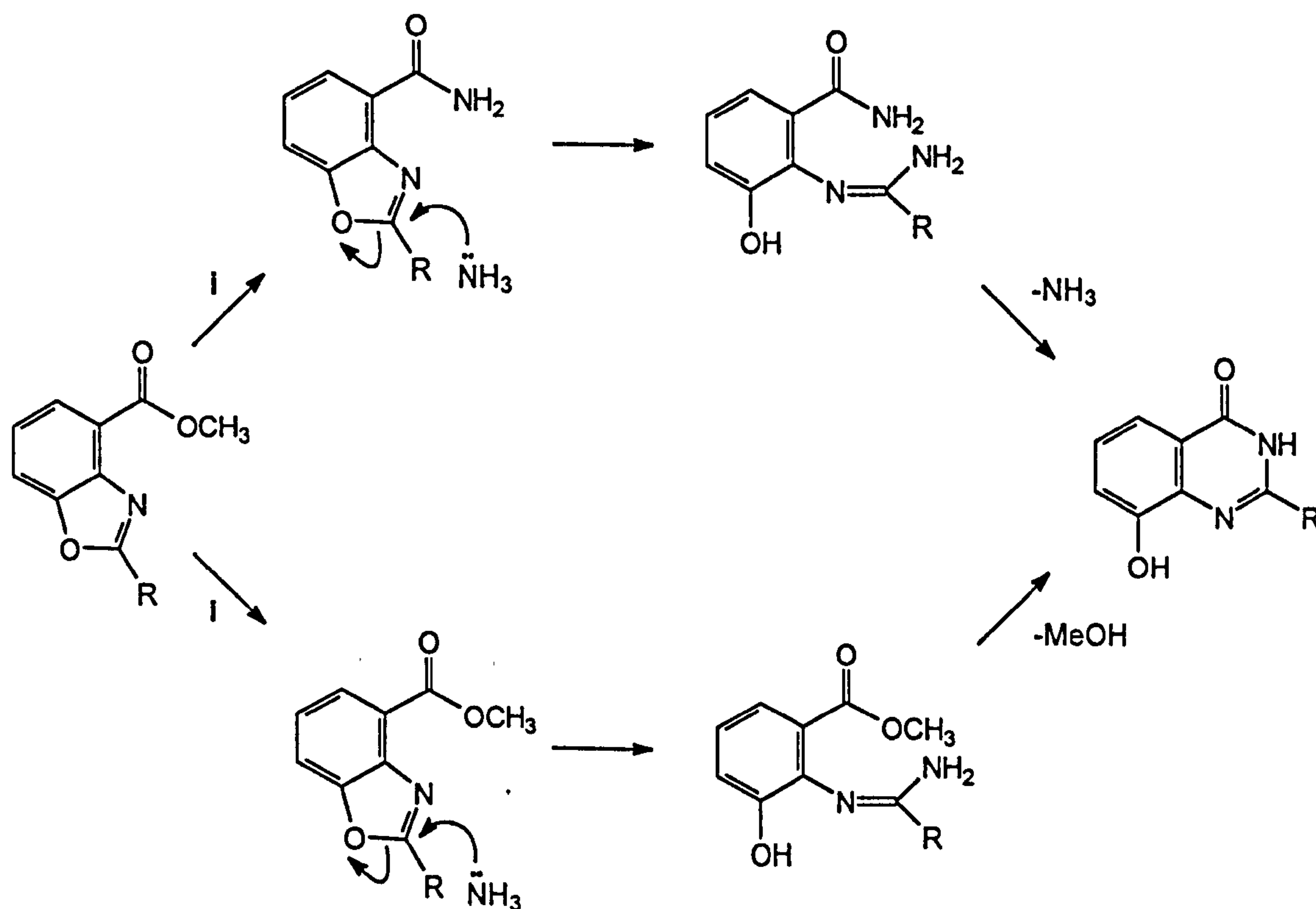
Previous studies, directed at the development of more selective and potent PARP inhibitors, identified a series of benzoxazole-4-carboxamides which displayed good activity.^{249,263} During the attempted synthesis of target compounds in the series, an unexpected rearrangement was observed which resulted in the formation of quinazolinone derivatives. These derivatives were assayed for inhibitory activity against PARP and were found to be extremely potent.²⁶³ The rearrangement was observed by chance, under conditions expected to convert methyl benzoxazole-4-carboxylates to the corresponding amides.^{249,263} *Scheme 5.3* shows the postulated mechanism for the rearrangement of the methyl benzoxazole-4-carboxylates.²⁶³ The initial step involves nucleophilic attack by ammonia at the C²-position of the benzoxazole, resulting in the ring-opening of the oxazole ring. Depending on whether aminolysis of the ester occurs prior to ring opening, subsequent intramolecular ring closure to form the quinazolinone would result in the elimination of either methanol or ammonia.²⁶³

The two quinazolinone derivatives identified from this rearrangement, 8-hydroxy-2-methylquinazolin-4-[3H]-one **37** (NU1025) and 8-hydroxy-2-(4'-nitrophenyl)quinazolin-4-[3H]-one **38** (NU1057), were found to have IC₅₀ values of 0.4 μ M and 0.2 μ M, respectively, when evaluated in a permeabilised cell assay.^{263,264} [IC₅₀ : inhibitory coefficient value, defined as the concentration of inhibitor required to reduce PARP activity to 50% of the control]. The excellent inhibitory activity of these compounds indicates that the quinazolinones are among the most potent PARP inhibitors known to date.

Scheme 5.3 Rearrangement of methyl benzoxazole-4-carboxylates to 8-hydroxyquinazolin-4-[3H]-ones²⁶³



37 R = CH₃
38 R = 4'-NO₂-C₆H₅

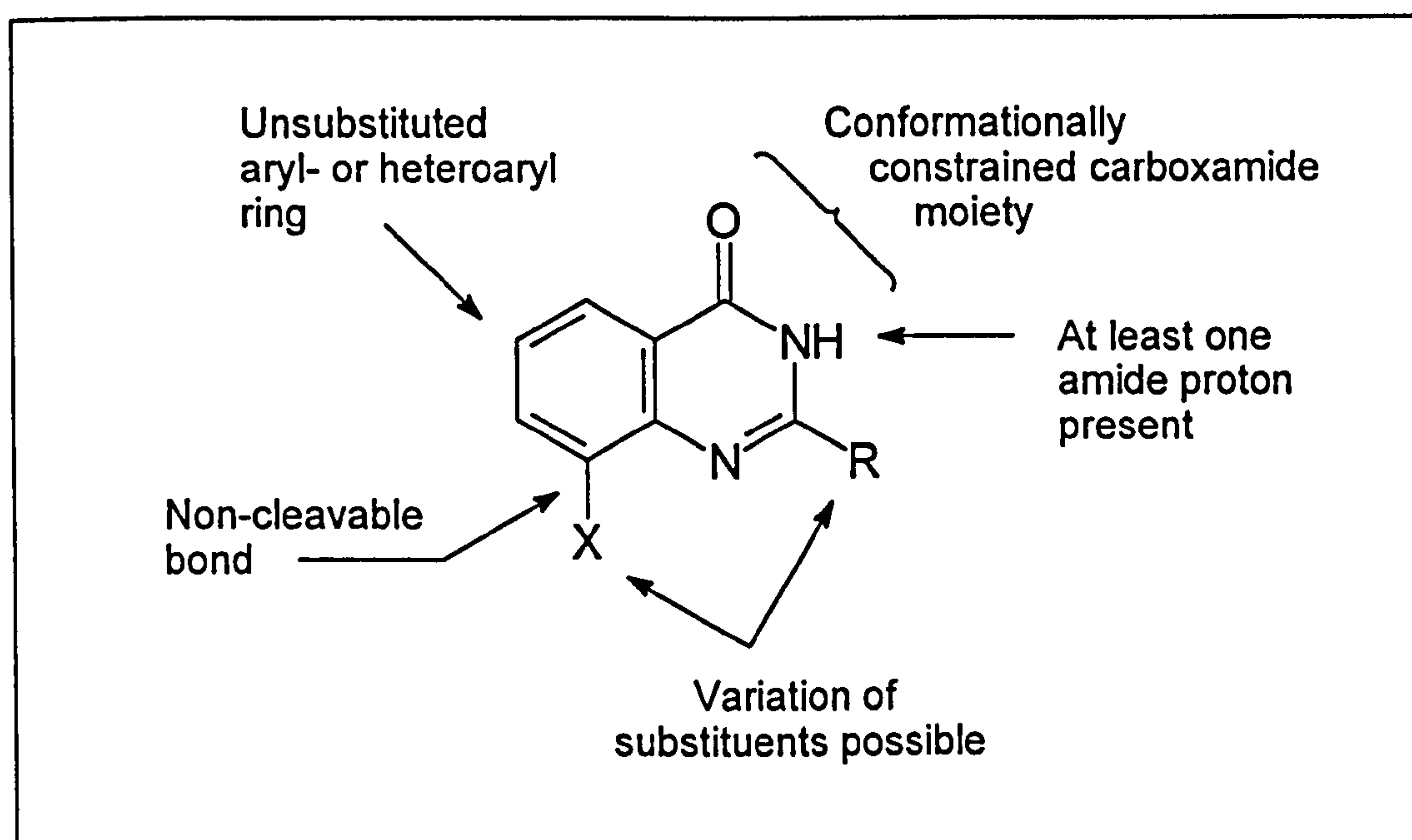


Reagents : i liquid NH₃, 20 bar pressure, 55 °C.

5.3.2 Quinazolinone Structure-Activity Relationships

The requisite structural features for efficient PARP inhibition (as discussed in Section 4.3) are incorporated into the basic quinazolinone architecture (see *Figure 5.3.2*).

Figure 5.3.2 Structural Features of Quinazolinone Inhibitors



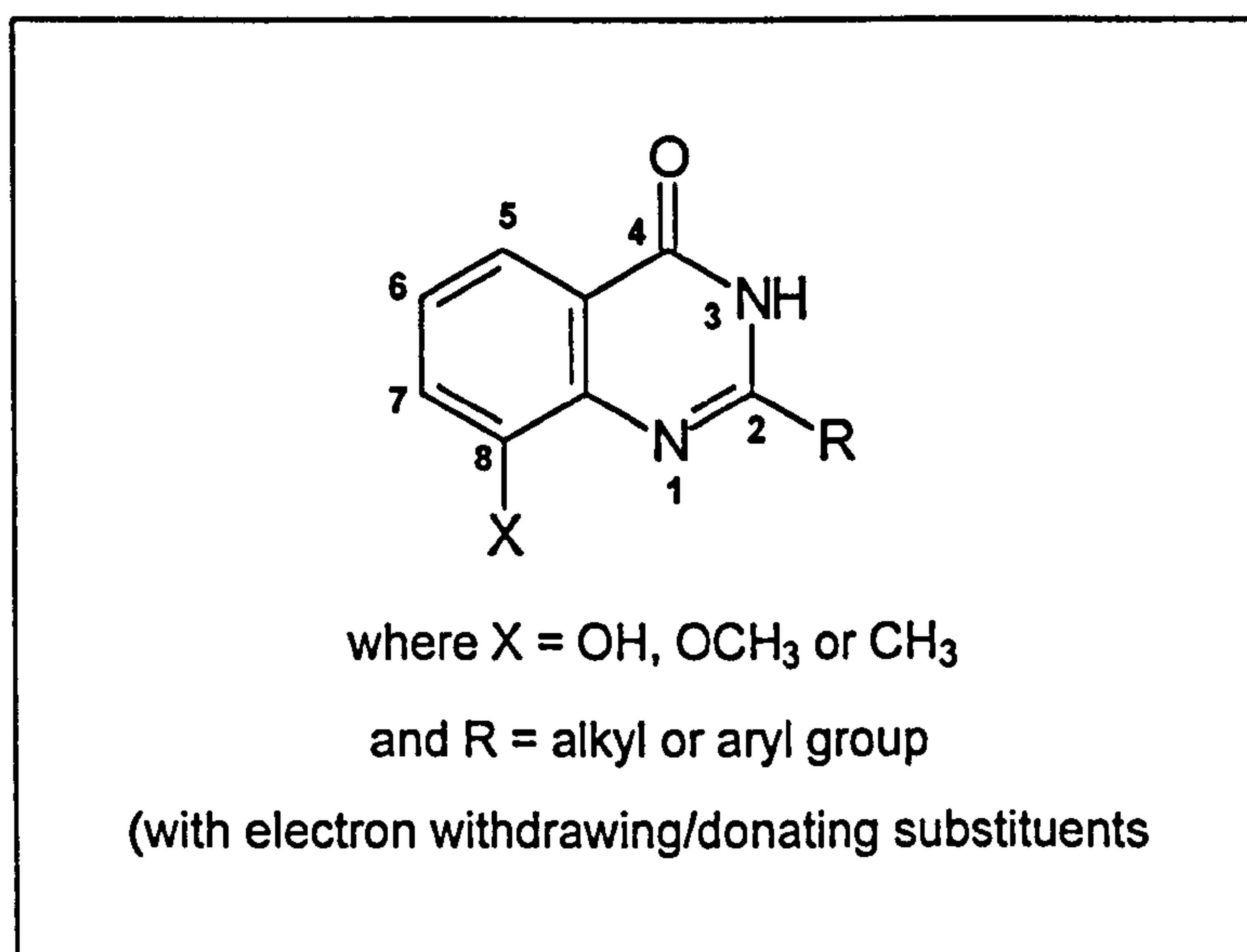
The compounds can be structurally elaborated by variation of the X and R substituents which may provide further information regarding structure-activity requirements.

5.4 Aims of Quinazolinone Research

Although quinazolinone formation was previously achieved through the rearrangement of methyl benzoxazole-4-carboxylates,²⁶³ these conditions do not represent an efficient route to novel PARP inhibitors. Biological evaluation of the two previously identified quinazolinones, 37 and 38, highlighted the potency of these compounds towards PARP and further quantities of NU1025 (37) were required for use in potentiation studies (see Chapter Seven, Section 7.4).

The initial aim of this research was to design an alternative, reproducible and high-yielding synthetic route to quinazolinone derivatives. A novel series of quinazolin-4-[3H]-ones of the following general structure was proposed (see *Figure 5.4*)

Figure 5.4 *Proposed general structure of quinazolinone PARP inhibitors*



Compounds would be synthesised with substituents acting as electron donors in the 8-position, in an attempt to establish whether or not the electronic nature of these substituents is of importance to enzyme-inhibitor interactions. Likewise, it was hoped that variation of the 2-substituent to include functional groups which were either electron-withdrawing or electron-donating in nature, would enable structure-activity relationships to be established for the quinazolinones. All purified quinazolinones would be biologically evaluated as potential inhibitors of PARP.

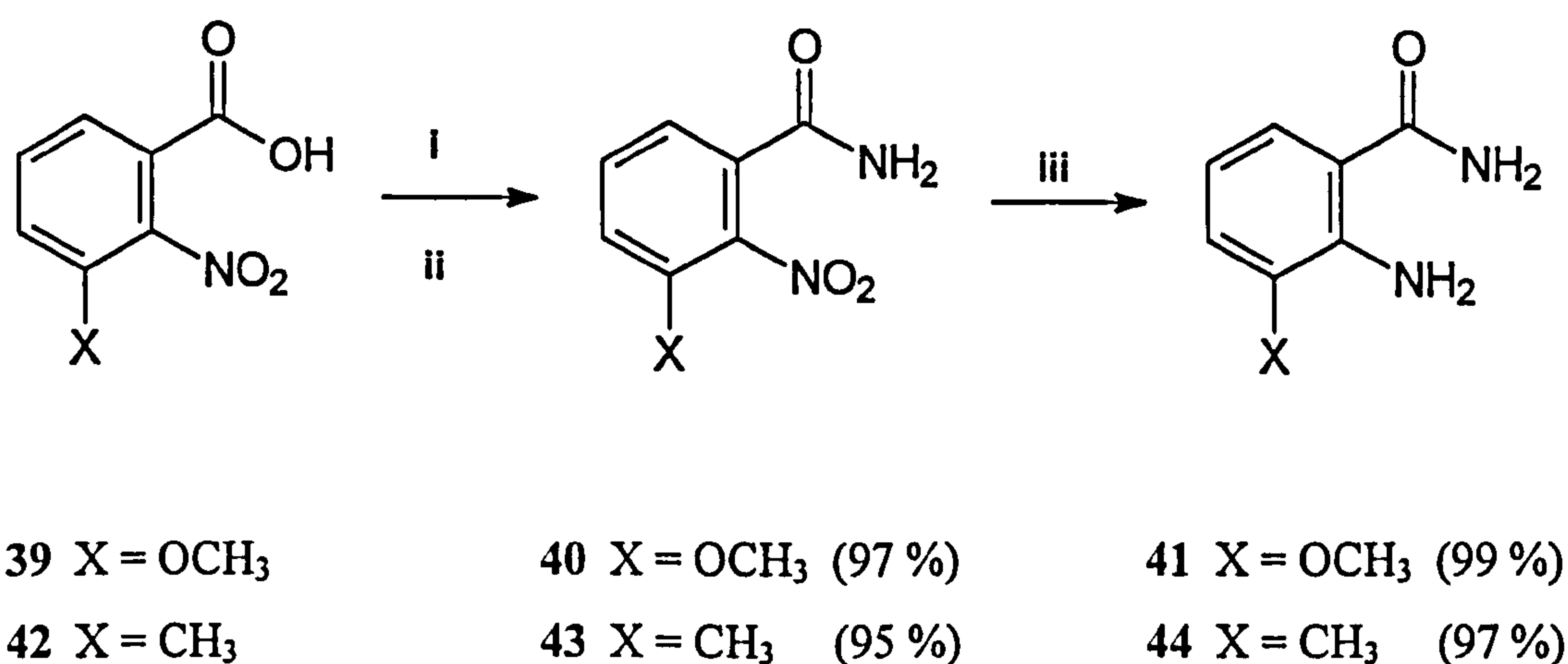
5.5 Syntheses of Quinazolinone PARP Inhibitors

5.5.1 Synthesis of 2-Alkylquinazolinones

The preparation of 2,8-disubstituted quinazolin-4-[3H]-ones was undertaken based upon the knowledge that quinazolinones can be formed from anthranilamide derivatives. It

was proposed that such derivatives could be synthesised from commercially available 2-nitrobenzoic acid derivatives. *Scheme 5.5.1.1* shows the synthetic route used to synthesise the requisite 3-methoxy-2-aminobenzamide **41** and 3-methyl-2-aminobenzamide **44**.

Scheme 5.5.1.1 *Synthesis of 3-methoxy-2-aminobenzamide **41** and 3-methyl-2-aminobenzamide **44***



Reagents : i SOCl₂/DMF, dry THF, 25 °C, ii NH₄OH, iii H₂/Pd/C, methanol.

Commercially available 3-methoxy-2-nitrobenzoic acid **39** was converted into the corresponding acid chloride using 1.5 mol. equivalents of thionyl chloride and a catalytic amount of DMF. The resulting 3-methoxy-2-nitrobenzoyl chloride was reacted further *in situ* with concentrated ammonia solution to give 3-methoxy-2-nitrobenzamide **40** in 97% yield. The product was found to be homogenous by thin layer chromatography (TLC) and analysis by proton nuclear magnetic resonance (¹H NMR) indicated that the crude product was suitable for subsequent reaction. The excellent yield of **40** obtained from this method of synthesis is comparable to the crude yield achieved in a previously

reported synthesis of the same compound.²⁶⁵ Compound 40 was readily reduced under an atmosphere of hydrogen in the presence of 10% palladium on activated carbon catalyst. 3-Methoxy-2-aminobenzamide 41 was obtained in 99% yield and was also used without further purification, on the basis of ¹H NMR analysis.

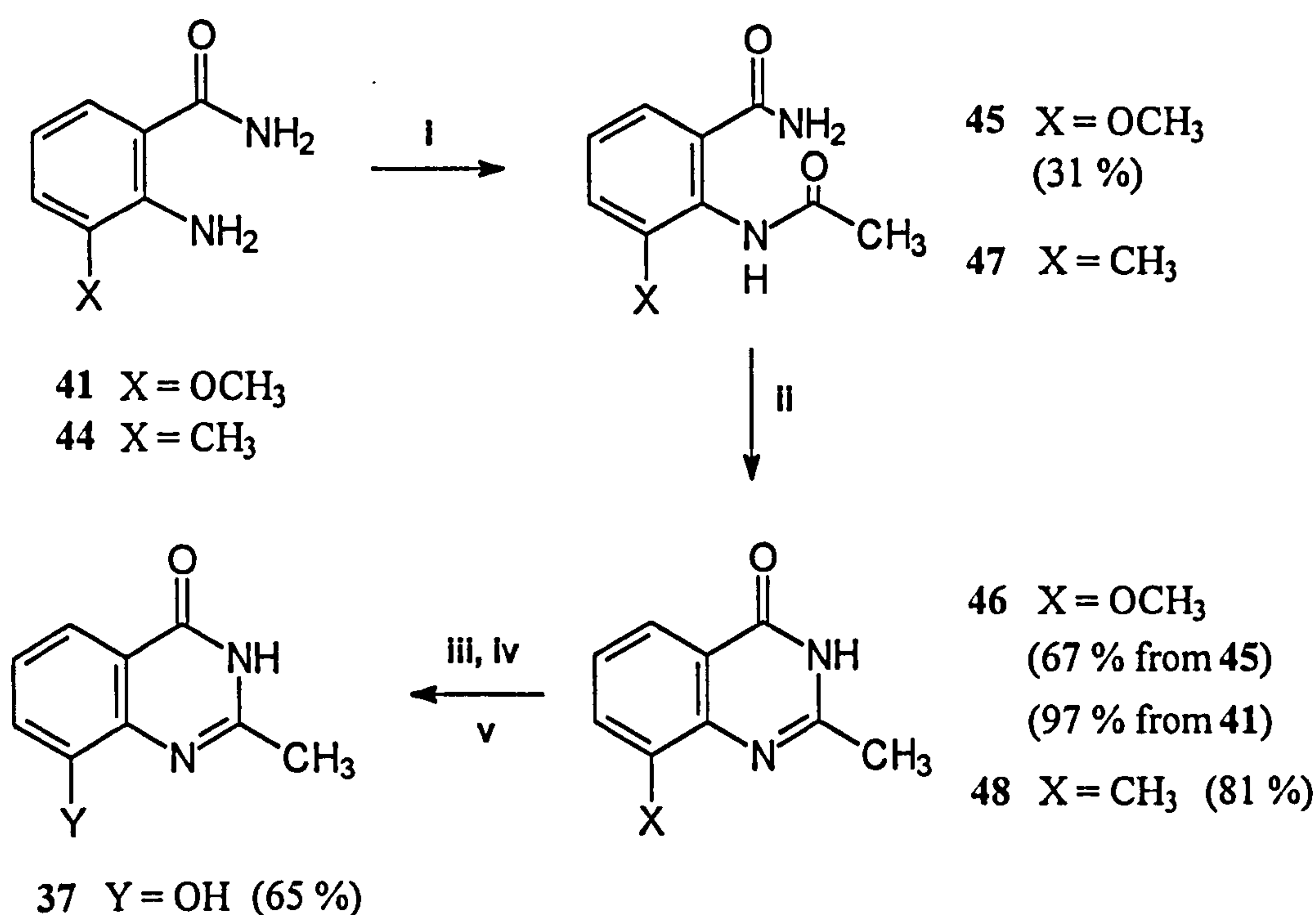
Following the high-yielding synthesis of compound 41, commercially available 3-methyl-2-nitrobenzoic acid 42 was subjected to the same reaction conditions to produce 3-methyl-2-nitrobenzamide 43 and 3-methyl-2-aminobenzamide 44, in 95% and 97% yields, respectively. The anthranilamide derivatives 41 and 44 were found to be slightly light-sensitive and large quantities of these compounds were accordingly stored under nitrogen in dark glass bottles.

Compound 41, when treated with 1.1 mol. equivalents of acetyl chloride in dry THF, in the presence of 1.3 mol. equivalents of pyridine, was acetylated at the 2-position to give 3-methoxy-2-*N*-acetylaminobenzamide 45. The structure of this compound was confirmed by ¹H NMR, infrared and mass spectral analysis and treatment of 45 with 2% aq. sodium hydroxide (NaOH) solution facilitated an intramolecular cyclisation reaction to give the ring-closed quinazolinone 46 (see *Scheme 5.5.1.2*). The yields of the individual steps were 31% for compound 45 and 67% for compound 46 to give an overall yield of only 20% with regard to 41. 8-Methyl-2-methylquinazolin-4-[3*H*]-one 46 was also synthesised in a 'one-pot' procedure. The crude intermediate 45, obtained after removal of the mother liquor, was simply redissolved in aq. NaOH solution and converted to 46. It was hoped that this would minimise loss through handling and purification of the intermediate and lead to a higher overall yield. In fact, yields of up to 97% were obtained for the one-pot method.

The synthesis of 2,8-dimethylquinazolin-4-[3*H*]-one 48 was carried out in order to establish whether the presence of an 8-substituent with reduced capacity to form hydrogen-bond interactions, would appreciably affect the activity of the compound. Compound 44 was converted to 2,8-dimethylquinazolin-4-[3*H*]-one 48 in 81% yield,

using the one-pot method. It was assumed that the intermediate would be compound 47, although this was not isolated.

Scheme 5.5.1.2 **Synthesis of 8-methoxy-2-methylquinazolin-4-[3H]-one 47 and 2,8-dimethylquinazolin-4-[3H]-one 48**



Reagents : i CH₃COCl, dry pyridine, dry THF, 25 °C, ii 2% aq. NaOH, 25 °C, iii BBr₃/DCM (1.0 M), reflux, iv 10% aq. NaOH, v aq. HCl.

Compound 46, 8-methoxy-2-methylquinazolin-4-[3H]-one, is the direct precursor of NU1025, 37. Demethylation of 46 was carried out following a literature method, using a 1.0 M solution of boron tribromide in dichloromethane (BBr₃ in DCM).²⁶⁶ The authors proposed the use of one equivalent of BBr₃/methoxy group and one equivalent/atom bearing lone pairs. Thus, a 1:4 mole ratio of 46:BBr₃ in DCM was gently heated under

reflux for 48 hours. The reaction generates the toxic gas methyl bromide and care must be taken to ensure that this by-product is efficiently trapped and destroyed. Following removal of the solvent from the reaction vessel, hydrolysis of the crude residue with 10% aq. NaOH solution gave the sodium salt of **37**. The desired phenolic product was obtained in 65% yield, after acidification of the reaction mixture with aq. HCl and recrystallisation.

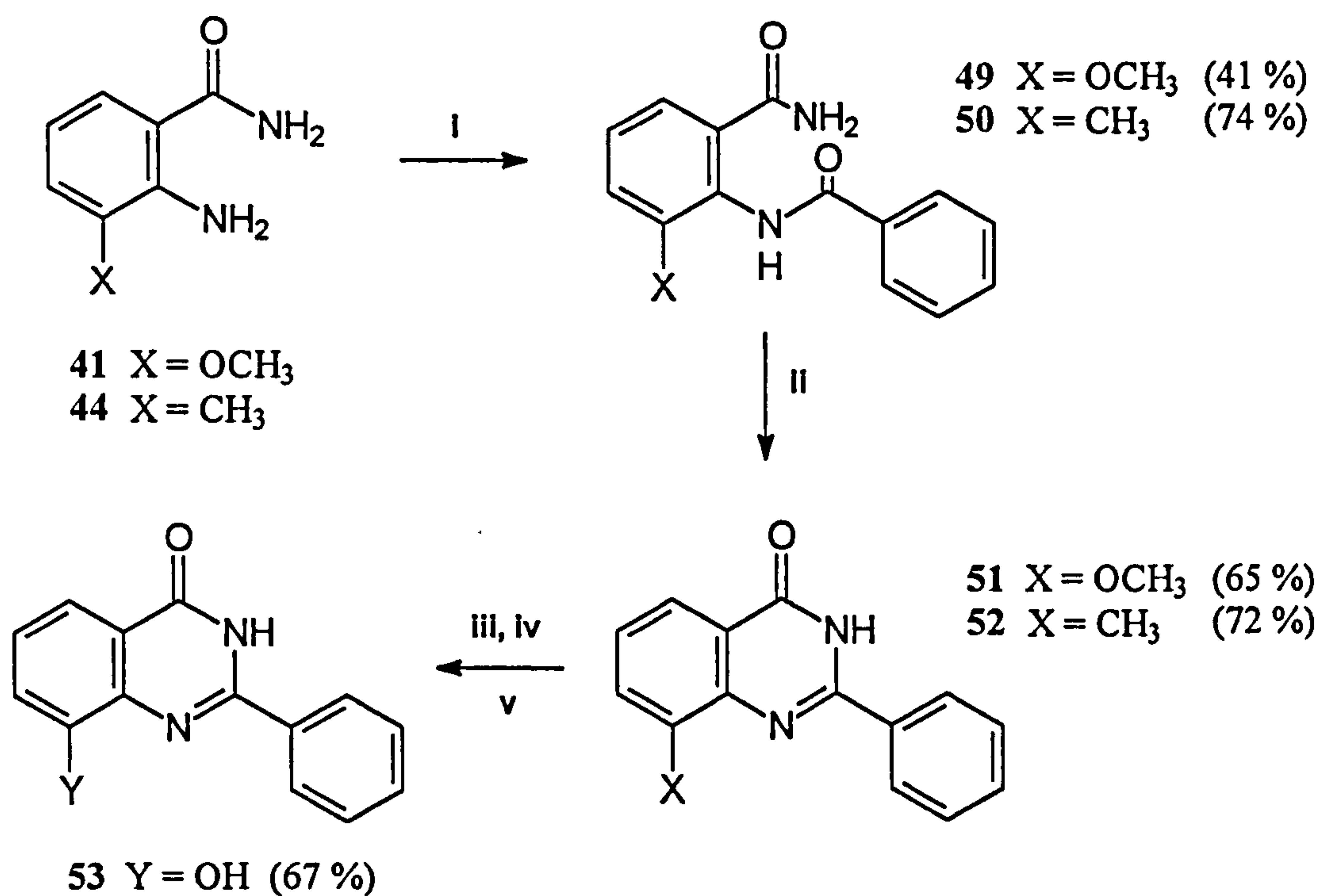
Analytical data obtained for the product, including elemental analysis, infra-red, NMR and mass spectra, indicated that the product was identical to **37**. Although it has already been established that this compound exhibits excellent PARP inhibitory activity *in vitro*, in order for further biological assays to be conducted, larger quantities of **37** were required, which were not available from the previous method. In view of the reasonable yield (61% overall yield with regard to the commercially available starting material), the method presented above provides a successful alternative synthesis of the target compound **37**. Since the two quinazolinones **46** and **48**, are the corresponding analogues of NU1025 **37**, they were also submitted for biological evaluation against PARP.

5.5.2 Synthesis of 2-Arylquinazolinones

It was necessary to synthesise 2-aryl derivatives for a comparison of structure-activity relationships with the 2-alkylquinazolinones (compounds **37**, **46** and **48**). It was proposed that the route employed to synthesise the 2-alkylquinazolinones should be used, as this would establish whether or not this could serve as a general method for the synthesis of 2-substituted quinazolinones.

8-Methoxy-2-phenylquinazolin-4-[3*H*]-one **51** and 8-methyl-2-phenylquinazolin-4-[3*H*]-one **52** were prepared as shown in *Scheme 5.5.2*, in 65% and 72% yields, respectively.

Scheme 5.5.2 Synthesis of 2-arylquinazolinones



Reagents : i C₆H₅COCl, dry pyridine, dry THF, 25 °C, ii 2% aq. NaOH, 25 °C, iii BBr₃/DCM (1.0 M), reflux, iv 10% aq. NaOH, v aq. HCl.

The *N*-benzoylaminobenzamides **49** and **50** were obtained using benzoyl chloride as the acylating agent and were readily cyclised in 2% aq. NaOH solution. 8-Hydroxy-2-phenylquinazolin-4-[3*H*]-one **53** was prepared by demethylation of **51** using the same method as before, and obtained in 67% yield. Compounds **51-53** were purified and submitted for biological evaluation. In view of the moderately high yields obtained for these compounds, the pathway appears to be applicable to the synthesis of 2-arylquinazolinones.

5.5.3 Synthesis of 2-(4'-Substituted-phenyl) Quinazolinones

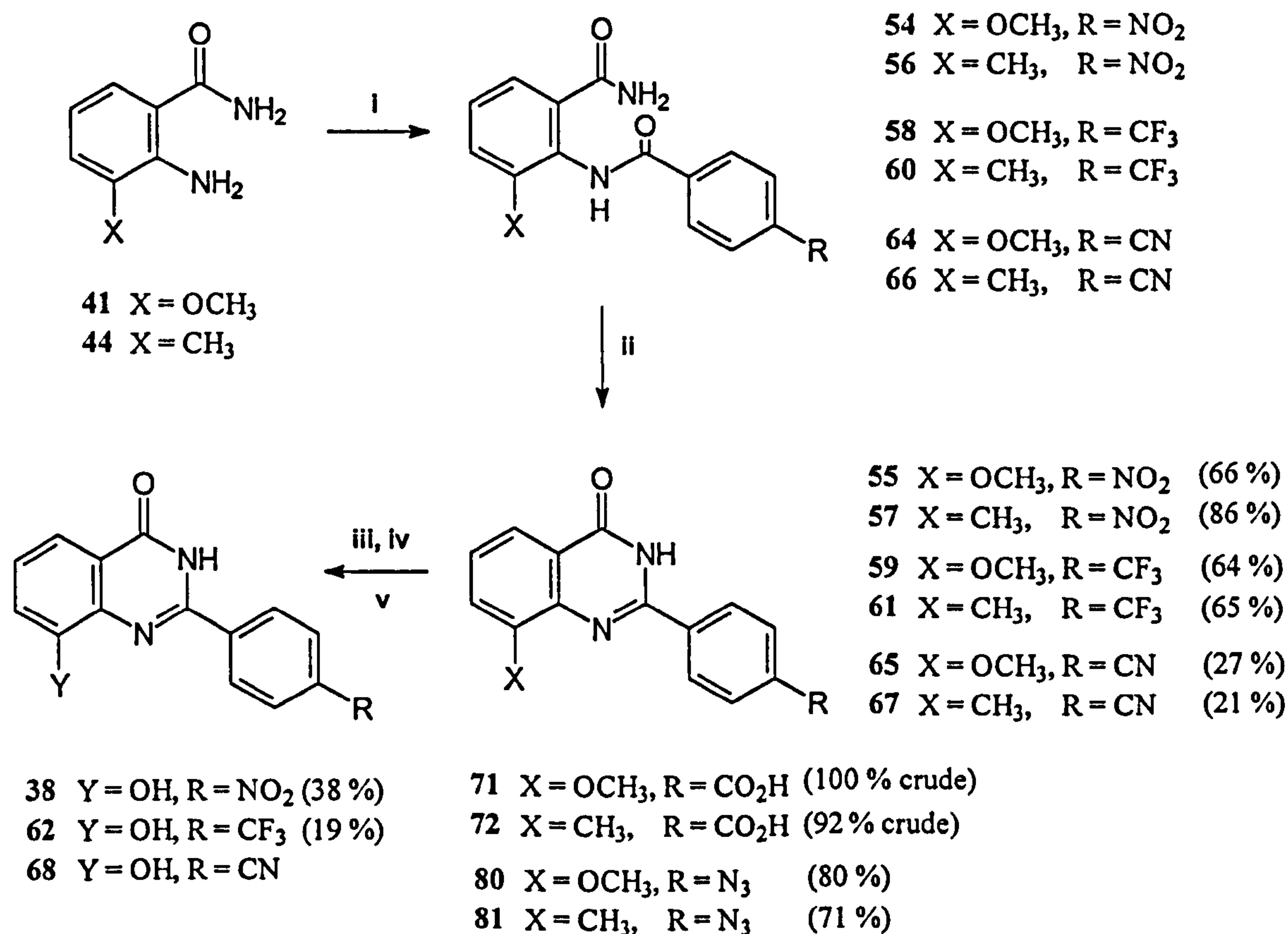
8-Hydroxy-2-(4'-nitrophenyl)quinazolin-4-[3*H*]-one **38** was previously identified as a highly potent PARP inhibitor.²⁶³ Syntheses in the quinazolinone series were extended to include derivatives bearing 4'-substituents on the aromatic ring in the 2-position. It was decided to vary the electronic nature of the 4'-substituent to include either electron-withdrawing or electron-donating functional groups.

5.5.3.1 Synthesis of Quinazolinones Bearing Electron-Withdrawing Substituents

Compounds bearing electron-withdrawing functionalities, including nitro, trifluoromethyl, cyano, carboxylate and azido groups, were synthesised according to *Scheme 5.5.3.1.1*. The synthesis of 8-methoxy-2-(4'-nitrophenyl)quinazolin-4-[3*H*]-one **55** was proposed from 3-methoxy-2-*N*-(4'-nitrobenzoyl)aminobenzamide **54**.^a The presence of the 4'-nitro substituent was expected to render the acyl carbonyl carbon more susceptible to nucleophilic attack and cyclisation. Thus, the cyclisation was initially attempted using 2% aq. NaOH solution as before. However, the main problem encountered was the insolubility of the acylated starting material **54** in the aqueous solvent. Although analogous reactions were carried out at room temperature, it was clear that more vigorous conditions were required to facilitate the ring closure of compound **54**. It was found that complete dissolution could be achieved by heating **54** in 10 % aq. NaOH at 100 °C. ¹H NMR data obtained from **55** were convincing although, again, the product was found to be virtually insoluble in various organic solvents, rendering purification of this compound problematic. The compound was finally recrystallised from aq. DMF at 100 °C. A hot filtration removed an insoluble cream product which was believed to be a salt. The required compound was obtained after concentration of the filtrate, in 66% yield.

^a Prepared by Mr R Davison, from 3-methoxy-2-aminobenzamide **41** and 4-nitrobenzoyl chloride.

Scheme 5.5.3.1.1 Synthesis of quinazolinones bearing electron-withdrawing substituents



Reagents : i RC₆H₄COCl, dry pyridine, dry THF, 25 °C, ii **54**, **56**: 10% aq. NaOH, 100 °C, **58**, **60**, **63**, **65**: 2% aq. NaOH, 25 °C, iii, BBr₃/DCM (1.0 M), iv 10% aq. NaOH, v aq. HCl.

8-Methyl-2-(4'-nitrophenyl)quinazolin-4-[3H]-one **57** was obtained using the one-pot procedure, in 86% yield after purification. As with compound **55**, more vigorous conditions were necessary to effect cyclisation, despite the presence of the strongly electron-withdrawing nitro group. Compound **57** was also poorly soluble in organic solvents and was purified as for **55**.

Demethylation of **55** afforded 8-hydroxy-2-(4'-nitrophenyl)quinazolin-4-[3H]-one **38**, in 38% yield after purification, a much lower yield than had been previously obtained for similar demethylation reactions. All purified (4'-nitrophenyl)quinazolinones were submitted for biological evaluation.

The trifluoromethyl derivatives, **59** and **61**, were prepared according to the one-pot procedure, using 4-trifluoromethylbenzoyl chloride as the acylating reagent. As with the 4'-nitrophenyl derivatives, the electron-withdrawing trifluoromethyl group should activate the compound towards cyclisation and, indeed, mild conditions were employed to facilitate ring closure of the intermediates **58** and **60**, to give **59** and **61** in 64% and 65% yields, respectively. Demethylation of **59** afforded 8-hydroxy-2-(4'-trifluoromethylphenyl)quinazolin-4-[3H]-one **62** in only 19% crude yield and the product proved difficult to purify owing to solubility problems. As a result, only compounds **59** and **61** were submitted for biological evaluation.

The quinazolinone series was further extended by the addition of the (4'-cyanophenyl)quinazolinones. The (4'-cyanophenyl)quinazolinones would be useful precursor compounds as the cyano substituent can be converted, using standard methodology, into a range of functionalities, including the amine, carboxylate, amide, aldehyde, ketone and tetrazole. 8-Methoxy-2-(4'-cyanophenyl)quinazolin-4-[3H]-one **65** and 8-methyl-2-(4'-cyanophenyl)quinazolin-4-[3H]-one **67** were prepared using 4-cyanobenzoyl chloride **63** as the acylating agent, which was readily synthesised from the corresponding acid.²⁵¹ Cyclisation to form the quinazolinones was effected with 2% aq. NaOH solution.

A general observation, concerning the poor solubility of some intermediates in both aqueous and organic solvents, was noted during the synthesis of quinazolinones bearing electron-withdrawing substituents. It appears that the stronger the electron-withdrawing substituent in the 4'-position, the poorer the solubility of the molecule in aqueous base.

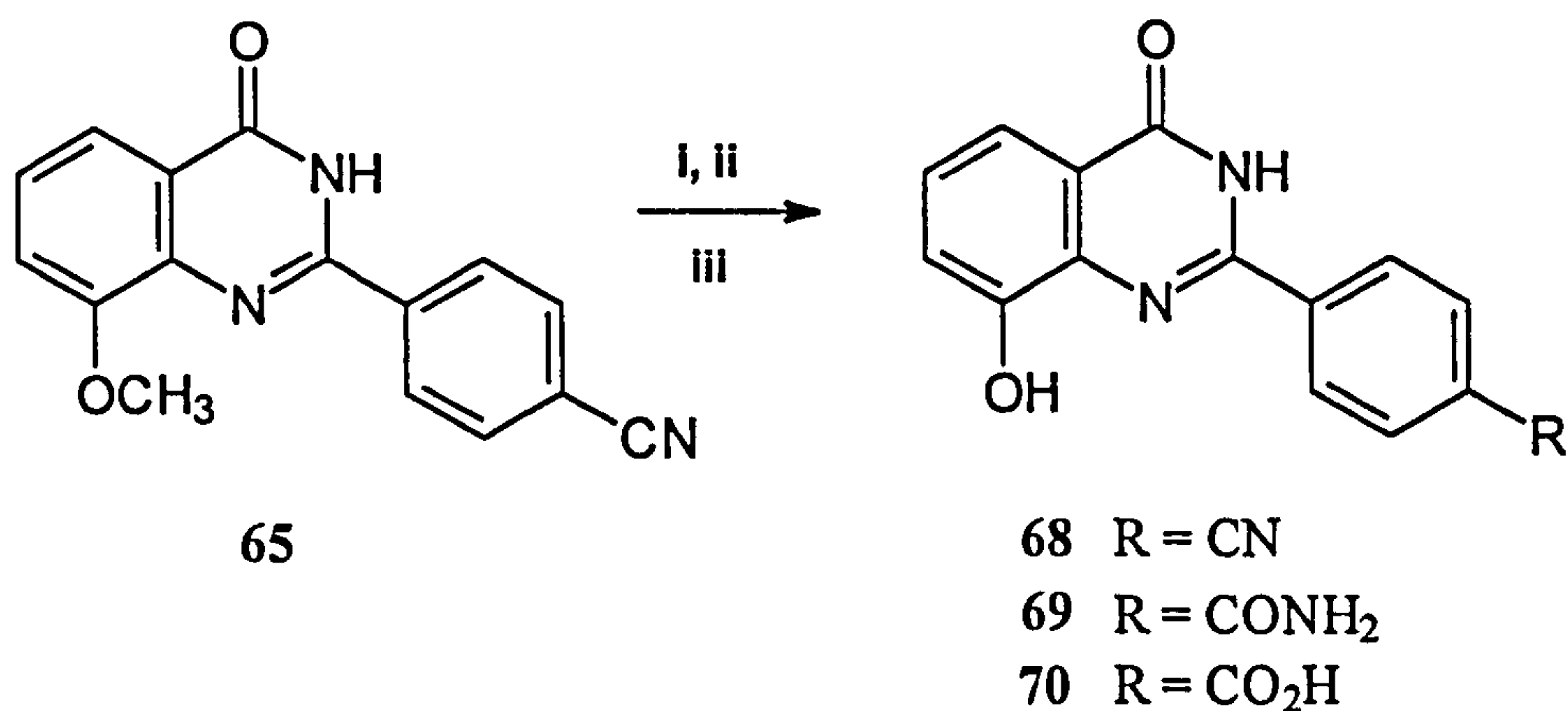
This was reflected by the marked insolubility of the (4'-nitrophenyl)quinazolinones and to a lesser extent, with the (4'-trifluoromethylphenyl)quinazolinones.

In the case of the (4'-cyanophenyl)quinazolinones, the yields of **65** and **67** were only 27% and 21%, respectively. These poor yields were mainly attributable to the insolubility of the *N*-acyl aminobenzamide precursors in aqueous media. The cyano group is almost as powerfully electron-withdrawing, both by resonance and inductive effects, as the nitro group but in light of the basic reaction conditions employed, the high reactivity of the cyano group poses a problem.

Identification of a second major product isolated during the purification of compound **67** by ¹H NMR, indicated that after 24 hours in 2% aq. NaOH solution at room temperature, a substantial amount of compound remained uncyclised. However, unlike with the (4'-nitrophenyl)quinazolinones, which are stable in basic media, the conditions used for cyclisation must be kept as mild as possible, otherwise the cyano group is hydrolysed to a carboxylate group. Therefore, in the case of the (4'-cyanophenyl)quinazolinones, an alternative reagent should be considered to avoid nitrile hydrolysis during the cyclisation.

8-Hydroxy-2-(4'-cyanophenyl)quinazolin-4-[3*H*]-one **68** was synthesised directly by demethylation, from the 8-methoxy precursor **65**. However, it is well known that nitriles react readily with Lewis acids to form non-ionic addition compounds, through the lone-pair electrons of the cyano group, and coordination compounds of nitriles and boron halides are well documented.²⁶⁷ Initially, the demethylation reaction was carried out over a 24 hour period when TLC analysis indicated the presence of three products. These products were isolated by column chromatography and identified by ¹H NMR analysis, as the required product **68**, the amide **69** and also the hydrolysis product **70** (see *Scheme 5.5.3.1.2*).

Scheme 5.5.3.1.2 **Demethylation of 8-methoxy-2-(4'-cyanophenyl) quinazolin-4-[3H]-one 65**



Reagents : i BBr₃/DCM (1.0 M), reflux, ii 10% aq. NaOH, iii aq. HCl.

The reaction was repeated and carefully monitored by TLC to detect by-product formation. It was found that after three hours, the amide was detected and despite the reaction being immediately worked up, a substantial amount of the amide was present. The reaction conditions for this particular reaction obviously need to be re-examined and an alternative non-Lewis acidic demethylating agent considered. Once again, the reactivity of the cyano group posed a problem when subjected to certain reagents in the general synthetic pathway. Although the by-products obtained may prove useful for biological evaluation, they should be synthesised under more controlled reaction conditions and isolated as major, not minor products.

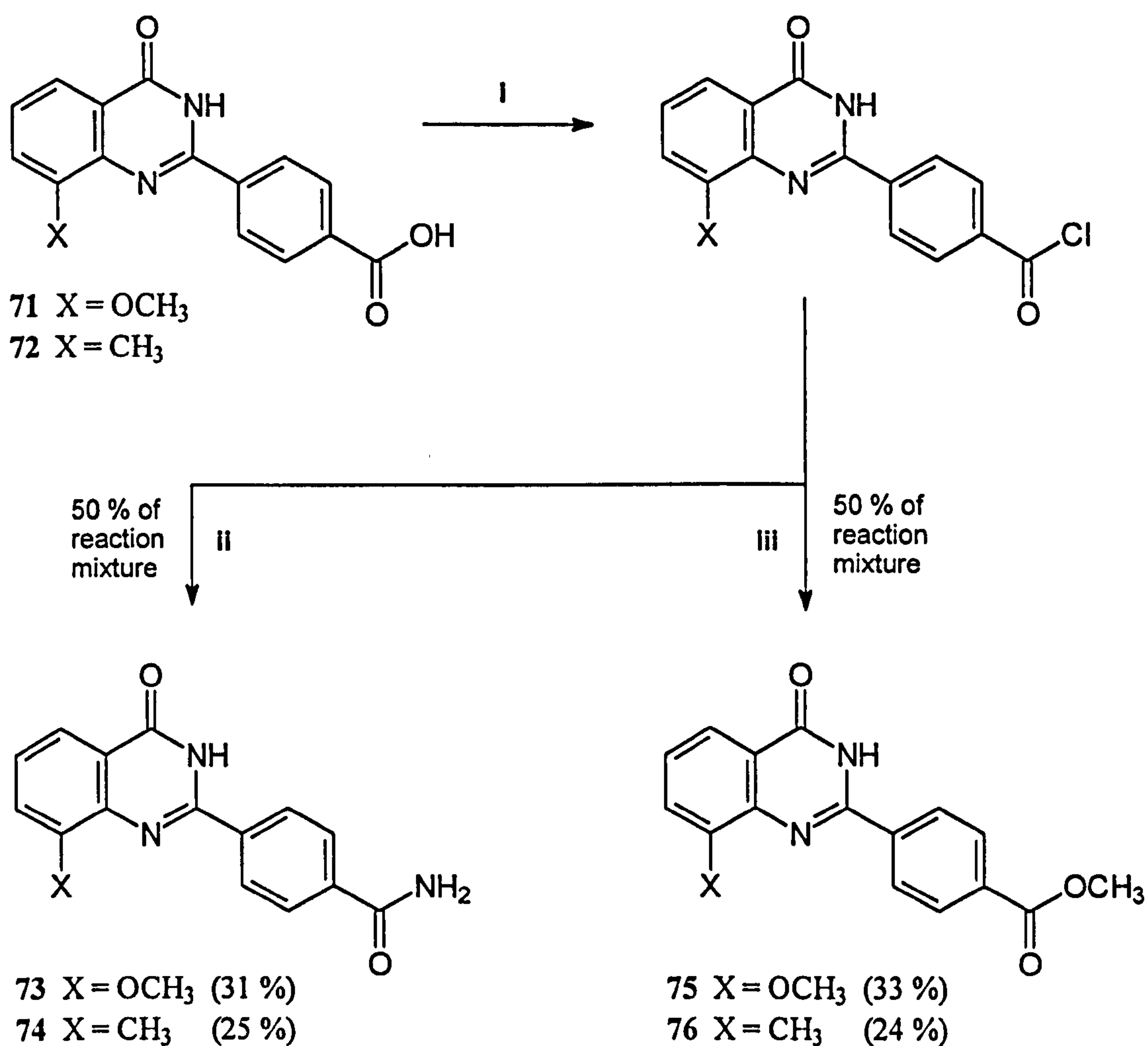
(4'-Carboxyphenyl)quinazolinones, **71** and **72**, were added to the quinazolinones series. Initially, the compounds were synthesised from the (4'-cyanophenyl)quinazolinones by base-catalysed hydrolysis.²⁶⁸ However, in view of the low isolated yields of the (4'-cyanophenyl)quinazolinones, it was deemed inappropriate to use these compounds as

starting materials. It was proposed that hydrolysis and cyclisation of 3-methoxy-2-*N*-(4'-cyanophenyl) aminobenzamide **64**, could be effected by using a stronger concentration of NaOH. This would ensure that any uncyclised material, which remains under mildly basic conditions, would also be converted to the (4'-carboxyphenyl)quinazolinone. This method could also be carried out as a one-pot procedure, starting from the appropriate 3-substituted-2-aminobenzamide.

Analytical data obtained for the (4'-carboxyphenyl)quinazolinones, **71** and **72**, indicated that these compounds had been isolated as salts. Data proved satisfactory with the exception of elemental analysis and mass spectra. It was hoped that the potency of the (4'-carboxyphenyl)quinazolinones would rival that of their isosteric counterparts the (4'-nitrophenyl)quinazolinones. Attempts to purify these compounds, however, proved unsuccessful and **71** and **72** were not submitted for biological evaluation.

It was hoped that the (4'-carboxyphenyl)quinazolinones would be sufficiently pure to allow the synthesis of compounds **73-76** (see *Scheme 5.5.3.1.3*). However, in each reaction, multiple products were observed which rendered purification of the products difficult. With the exception of compound **76**, satisfactory combustion analysis results were not obtained and only compound **76** was submitted for biological evaluation. It was proposed that a side reaction may be occurring between the quinazolinones and thionyl chloride.

Scheme 5.5.3.1.3 Synthesis of (4'-carboxamidophenyl)quinazolinones **73** and **74**, and (4'-carboxymethylphenyl)quinazolinones, **75** and **76**



Reagents : i SOCl₂/DMF, dry THF, 25 °C, ii NH₄OH, iii dry methanol.

To investigate the possibility of a side reaction taking place, a model reaction was carried out using 8-methoxy-2-phenylquinazolin-4-[3H]-one **51**.

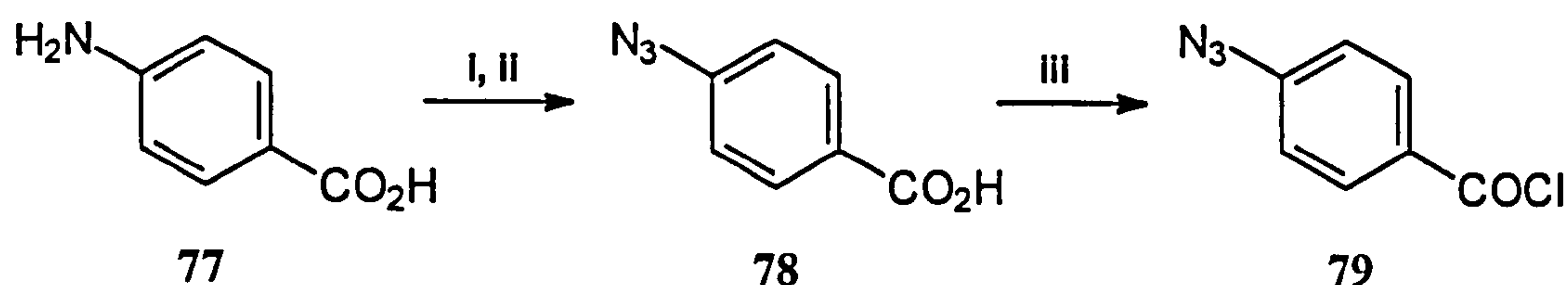
Compound **51** was treated with:

- i) SOCl_2 (2 eq.), DMF (catalytic), dry THF, 25 °C, and
- ii) SOCl_2 , at reflux.

After one hour, no starting material was evident in either reaction, indicating that thionyl chloride may not be compatible with the basic quinazolinone structure. A multi-spot product was detected by TLC and no product was isolated. A possible solution to this problem would be to convert the (4'-carboxyphenyl)quinazolinone to a mixed anhydride, as this could be carried out under much milder conditions. The mixed anhydrides could then be reacted with a variety of amines or alcohols to give the corresponding substituted amides or esters.

The (4'-azidophenyl)quinazolinone **80** was synthesised in 80 % yield, following the usual synthetic route.^b The acylating species, 4-azidobenzoyl chloride **79**, was readily prepared according to *Scheme 5.5.3.1.4*.

Scheme 5.5.3.1.4 Preparation of 4-azidobenzoyl chloride



Reagents : i NaN_3 , NaNO_2 , 5M HCl, <5 °C, ii SOCl_2 , DMF, dry THF, 25 °C.

8-Methyl-2-(4'-azidophenyl)quinazolin-4-[3H]-one **81** was prepared in a similar manner to **80** in 71% overall yield.^c The (4'-azidophenyl)quinazolinones **80** and **81** are useful

^b Prepared by Miss H. C. Mullen, vocational research student.

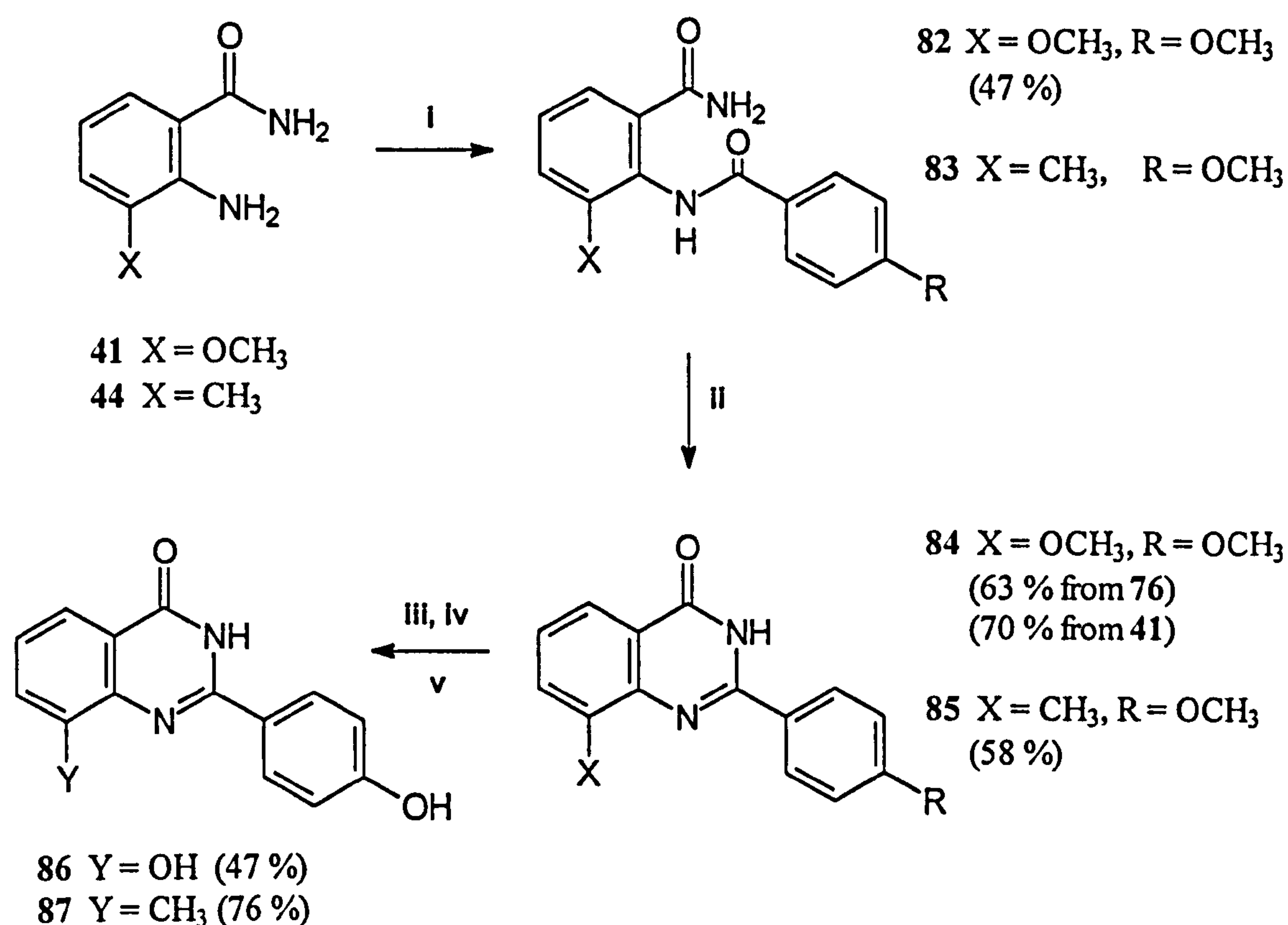
^c Prepared by Mr R. Davison.

precursor compounds, as the azido group can be converted to a range of functionalities. However, the lack of time available did not allow subsequent reactions to be carried out.

5.5.3.2 Synthesis of Quinazolinones Bearing Electron-Donating Substituents

Compounds bearing electron-donating functionalities in the 2-aryl ring, such as methoxy and hydroxy groups, were synthesised according to *Scheme 5.5.3.2.1*.

Scheme 5.5.3.2.1 Synthesis of quinazolinones bearing electron-donating substituents

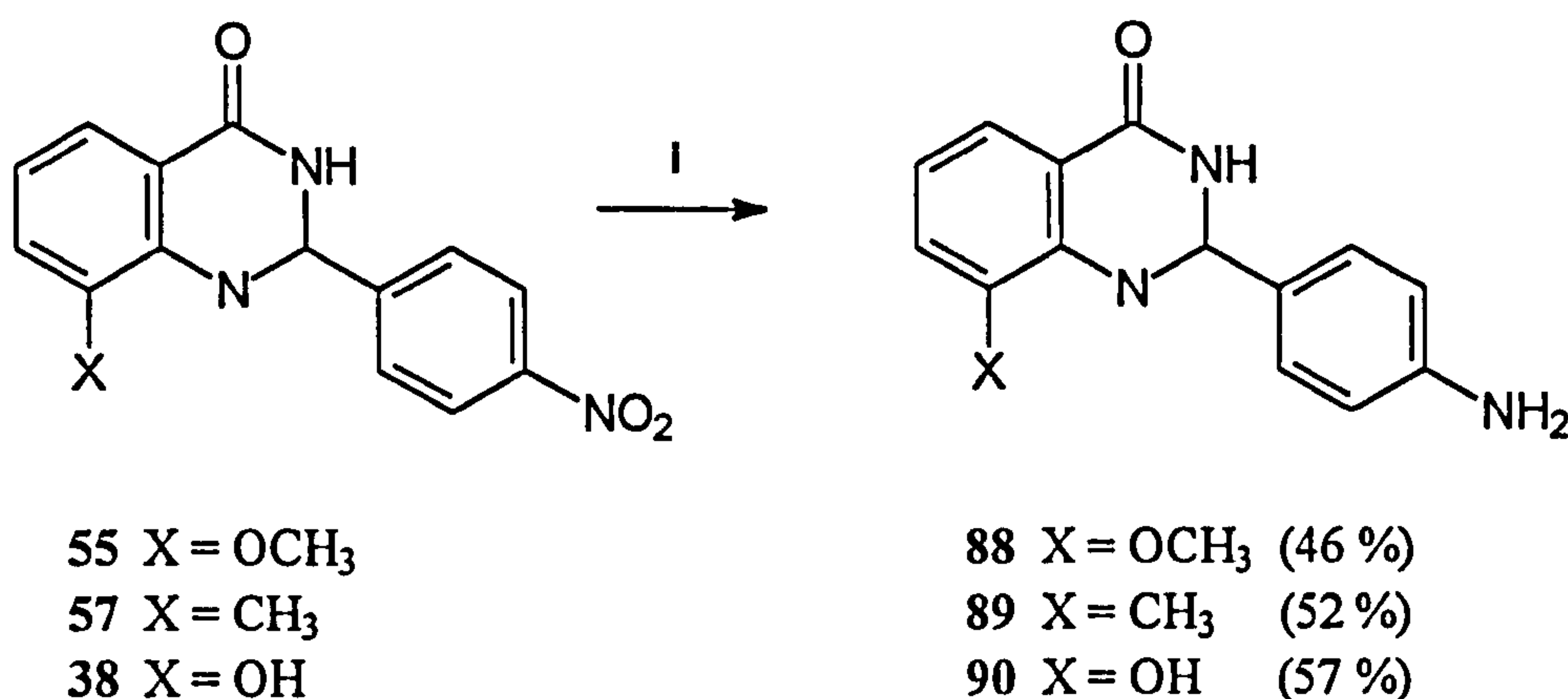


Reagents : i RC₆H₄COCl, dry pyridine, DMAP (5 mol. %), dry THF, 25 °C, ii 10% aq. NaOH, reflux, iii BBr₃/DCM (1.0 M), reflux, iv 10% aq. NaOH, 25 °C, v aq. HCl.

Compound 84 was also prepared without isolation of the intermediate 82, as was 8-methyl-2-(4'-methoxyphenyl)quinazolin-4-[3H]-one 85, in 70% and 58% yields, respectively, and both compounds were submitted for biological evaluation.

8-Hydroxy-2-(4'-hydroxyphenyl)quinazolin-4-[3H]-one 86 and 8-methyl-2-(4'-hydroxyphenyl)quinazolin-4-[3H]-one 87 were obtained by demethylation of compounds 84 and 85, respectively. The chemical similarity between the two methoxy substituents present in 84 led to both groups being cleaved by BBr₃. Clearly, the synthetic route employed in the general synthesis of the quinazolin-4-[3H]-ones was not appropriate for the synthesis of 8-hydroxy-2-(4'-methoxyphenyl)quinazolin-4-[3H]-one or 8-methoxy-2-(4'-hydroxyphenyl)quinazolin-4-[3H]-one and an alternative route using suitable protecting group chemistry is required. Compounds 86 and 87 incorporate hydroxyl moieties into their structures and would thus be suitable candidates for prodrug modification.

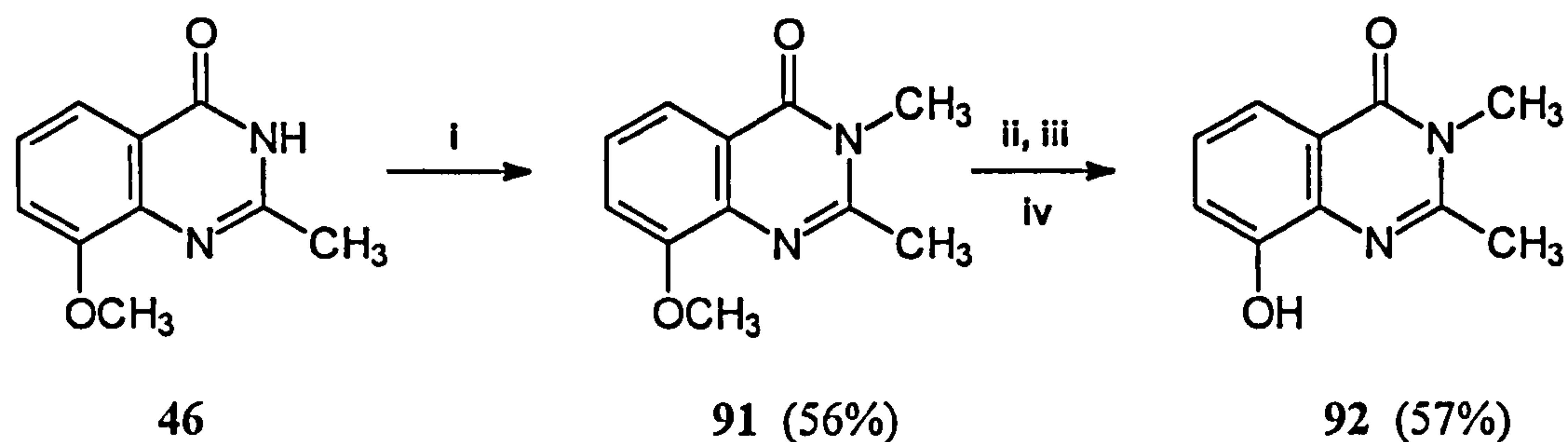
The (4'-aminophenyl)quinazolinones were synthesised directly from the corresponding (4'-nitrophenyl)quinazolinone derivatives, by palladium-catalysed reduction of the aromatic nitro groups (*Scheme 5.5.3.2.2*). The moderate yields obtained were attributed to the insolubility of the starting materials in methanol. Unlike the derivatives bearing electron-withdrawing substituents, solubility in organic solvents was found to be adequate with quinazolinones bearing electron-donating substituents, and these were purified by recrystallisation from methanol.

Scheme 5.5.3.2.2 Synthesis of (4'-aminophenyl)quinazolinones

Reagents : i H₂/Pd/C, methanol, 25 °C.

5.6 Synthesis of Control Compounds

It has previously been shown that methylation of the *N*³-position of NU1025 (37), results in a significant loss of PARP inhibitory activity.^{263,264} The *N*³-methylated quinazolinones 91 and 92 were synthesised as control compounds for use in PARP inhibition studies. Compound 91 was prepared in 56% yield, by treating 8-methoxy-2-methylquinazolin-4-[3*H*]-one 46 with methyl iodide, in the presence of potassium carbonate, using acetonitrile as solvent.²⁶³ Compound 92 was synthesised in 57% yield, by demethylation of 91 (*Scheme 5.6*).

Scheme 5.6 *Synthesis of N³-methylquinazolinones*

Reagents : i CH_3I , K_2CO_3 , dry MeCN, reflux, ii BBr_3/DCM (1.0 M), reflux, iii 10% aq. NaOH, iv HCl.

5.7 **Limitations of Quinazolinone Synthesis**

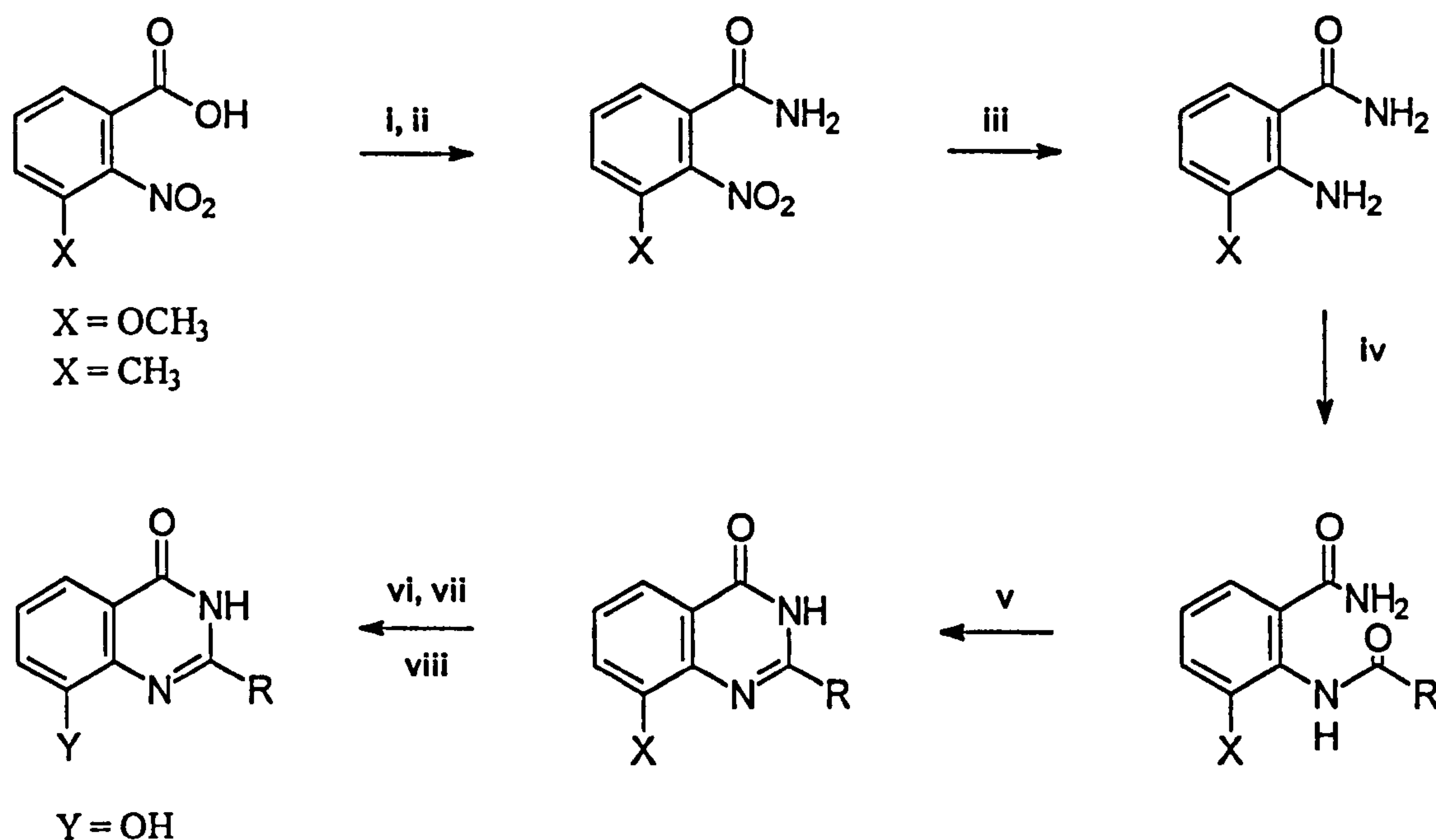
The synthetic pathway used to synthesise the majority of the quinazolinone derivatives is shown in *Scheme 5.7*.

The above scheme works efficiently in practice but a number of limitations are apparent, due mainly to the poor stability of functional groups under the reaction conditions employed, *e.g.*

- i) hydrolysis of the (4'-cyanophenyl)quinazolinones under basic conditions,
- ii) formation of nitrile and trihalide complexes with the Lewis-acidic reagent, BBr_3
- iii) incompatibility of quinazolinones with thionyl chloride.

There are a number of compounds in the current series which are inaccessible by the above route and alternative methods need consideration.

Scheme 5.7 General synthetic pathway for quinazolin-4-[3H]-ones



Reagents : i SOCl_2/DMF , dry THF, 25 °C, ii NH_4OH , iii $\text{H}_2/\text{Pd/C}$, methanol, iv RCOCl , dry pyridine, dry THF, 25 °C, v aq. NaOH .

If $\text{X} = \text{OCH}_3$: vi BBr_3/DCM (1.0 M), reflux, vii 10% aq. NaOH , viii aq. HCl .

5.8 Design of Quinazolinone Prodrugs

The design of quinazolinone prodrugs was undertaken in an attempt to circumvent the problems associated with the insolubility of the quinazolinone derivatives in aqueous media. There are a number of factors which should be considered when selecting a suitable candidate compound for prodrug modification:

- the parent compound should exhibit potent inhibitory activity *in vitro*;
- an appropriate functional group for prodrug modification, such as hydroxyl or amino, should be present in the parent compound;

- iii) the prodrug should be biologically inactive, chemically stable and readily soluble in aqueous media;
- iv) the prodrug should be non-toxic, as should the prodrug modification itself, when cleaved from the parent compound *in vivo*;
- v) the prodrug should undergo facile metabolic activation *in vivo* to the parent compound (normally *via* enzymatic cleavage);
- vi) consideration must also be given to the bioavailability of the prodrug, as it should not be readily eliminated from the body.

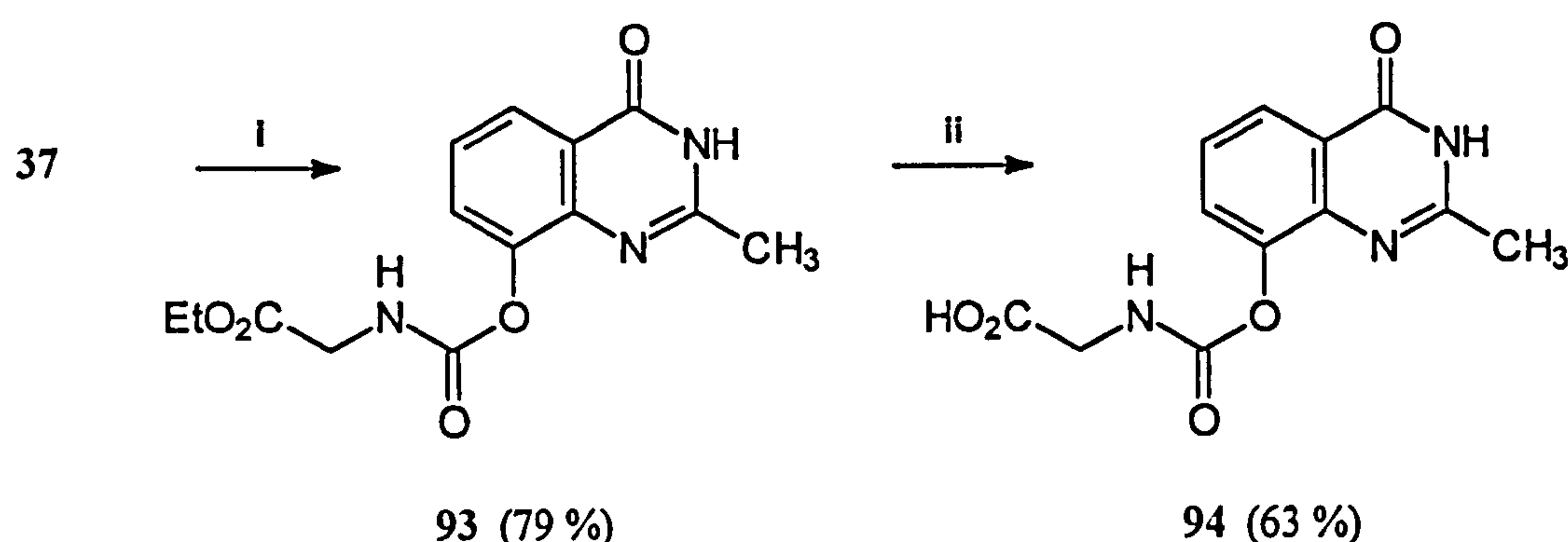
NU1025 37, was chosen for development as a potential prodrug, since the compound exhibits excellent *in vitro* PARP inhibitory activity and contains a phenolic hydroxyl group amenable to prodrug modification.

5.8.1 Synthesis of Carbamate Ester Prodrugs

Several drugs containing phenolic hydroxyl groups are known to undergo extensive first-pass metabolism, mainly by way of sulfation, methylation or glucuronidation of the phenolic moieties.²⁶⁹ Phenyl carbamates have been used as prodrug modifications in an attempt to reduce the extent of first-pass metabolism of the vulnerable phenolic group.^{270,271} Carbamate ester modifications have proved to be extremely successful with a number of pharmaceuticals, including physostigmine, neostigmine and carbachol.²⁷² The anticancer agent, combretastatin A4 was converted to a glycine carbamate prodrug, which served to greatly enhance the overall aqueous solubility of the molecule and provided a safe vehicle for the administration of the drug *in vivo*.²⁷³

It was proposed that a similar derivative could be synthesised using NU1025 (37), as the parent compound. It was hoped that the glycine carbamate derivative of 37 could be isolated as a water-soluble salt and metabolic activation of the prodrug would result in the elimination of the non-toxic glycine moiety. Compound 37 was converted to the glycine carbamate ethyl ester 93, using 1.1 mol. equivalents of ethyl isocyanatoacetate, in the presence of dry triethylamine (*Scheme 5.8.1.1*).

Scheme 5.8.1.1 Synthesis of NU1025-glycine carbamate



Reagents : i EtO₂CCH₂NCO, dry THF, dry NEt₃, 25 °C, ii THF:0.5 M H₂SO₄ (1:1 ratio)

The reaction was found to be high-yielding and clean, with a simple recrystallisation giving the protected carbamate **93** in 79% yield. Deprotection of **93** was effected with a 1:1 ratio of THF and 0.5 M H₂SO₄, to give the target glycine carbamate **94** in 63% yield.

Promising ¹H NMR data were obtained for the deprotected product and the compound was found to readily dissolve in aqueous sodium bicarbonate solution, indicating the formation of a sodium salt. However, ¹H NMR analysis of the product isolated from alkaline solution revealed that instead of forming a chemically stable salt, the carbamate ester was unstable and underwent hydrolysis to give the parent quinazolinone **37**. Numerous investigations concerning the stability of carbamate esters towards alkaline hydrolysis have been carried out.^{272,274,275} It has been proposed that the hydrolysis of aryl carbamates formed from isocyanate derivatives, proceeds *via* an isocyanate intermediate (*i.e.* the formation and decomposition reactions share a common mechanistic pathway).²⁷⁵

With regard to producing a water-soluble quinazolinone which would undergo enzymatic cleavage in plasma, the carbamate ester was considered too unstable, as its degradation to

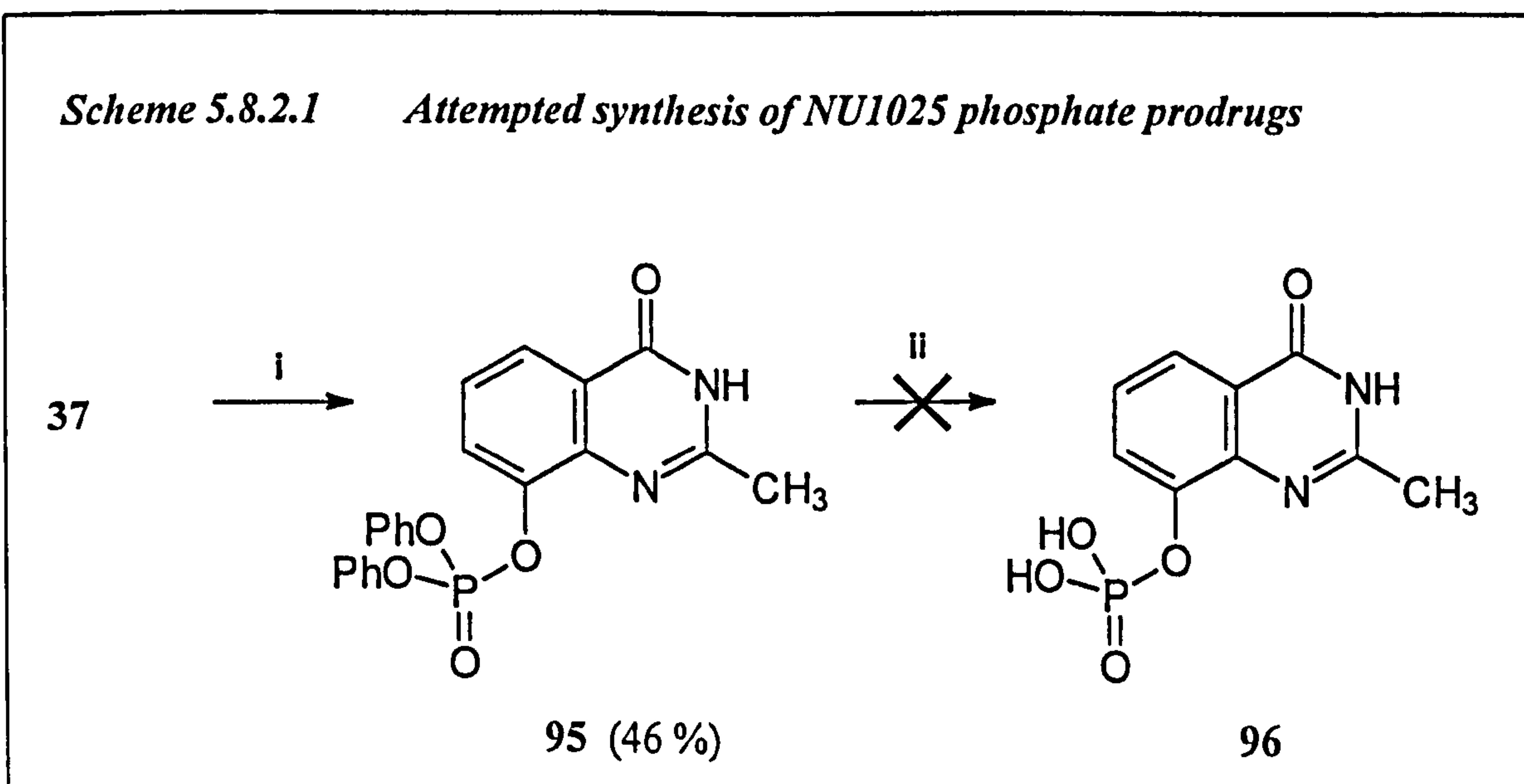
the parent compound was pH and not enzyme-dependent. It was decided to abandon this type of prodrug modification in favour of more chemically stable derivatives.

5.8.2 Synthesis of Phosphate Prodrugs

Certain phosphate derivatives make ideal prodrugs, as they can be metabolically activated by non-specific, endogenous phosphatases.²⁷⁶ The phosphate prodrug modification has been applied successfully to a number of anticancer agents, including combretastatin A4,²⁷⁶ Taxol,²⁷⁷ and Etoposide.²⁷⁸ In all cases, the introduction of a phosphate group greatly enhanced aqueous solubility and resulted in the formation of chemically stable prodrug derivatives.

Again, NU1025 (37), was used as the parent compound for prodrug modification. Using the same method employed to convert etoposide to etopophos, 37 was treated with 1.1 mol. equivalents of commercially available diphenyl chlorophosphate, in the presence of *N,N*-diisopropylethylamine [EtN(ⁱPr)₂], using acetonitrile as solvent²⁷⁹ (see *Scheme 5.8.2.1*). The resulting diphenyl phosphate ester 95 was obtained in 46% yield.

Scheme 5.8.2.1 Attempted synthesis of NU1025 phosphate prodrugs



Reagents : i dry EtN(ⁱPr)₂, (PhO)₂P(O)Cl, dry MeCN, 25 °C, ii PtO₂/H₂, 45-50 psi, ethanol.

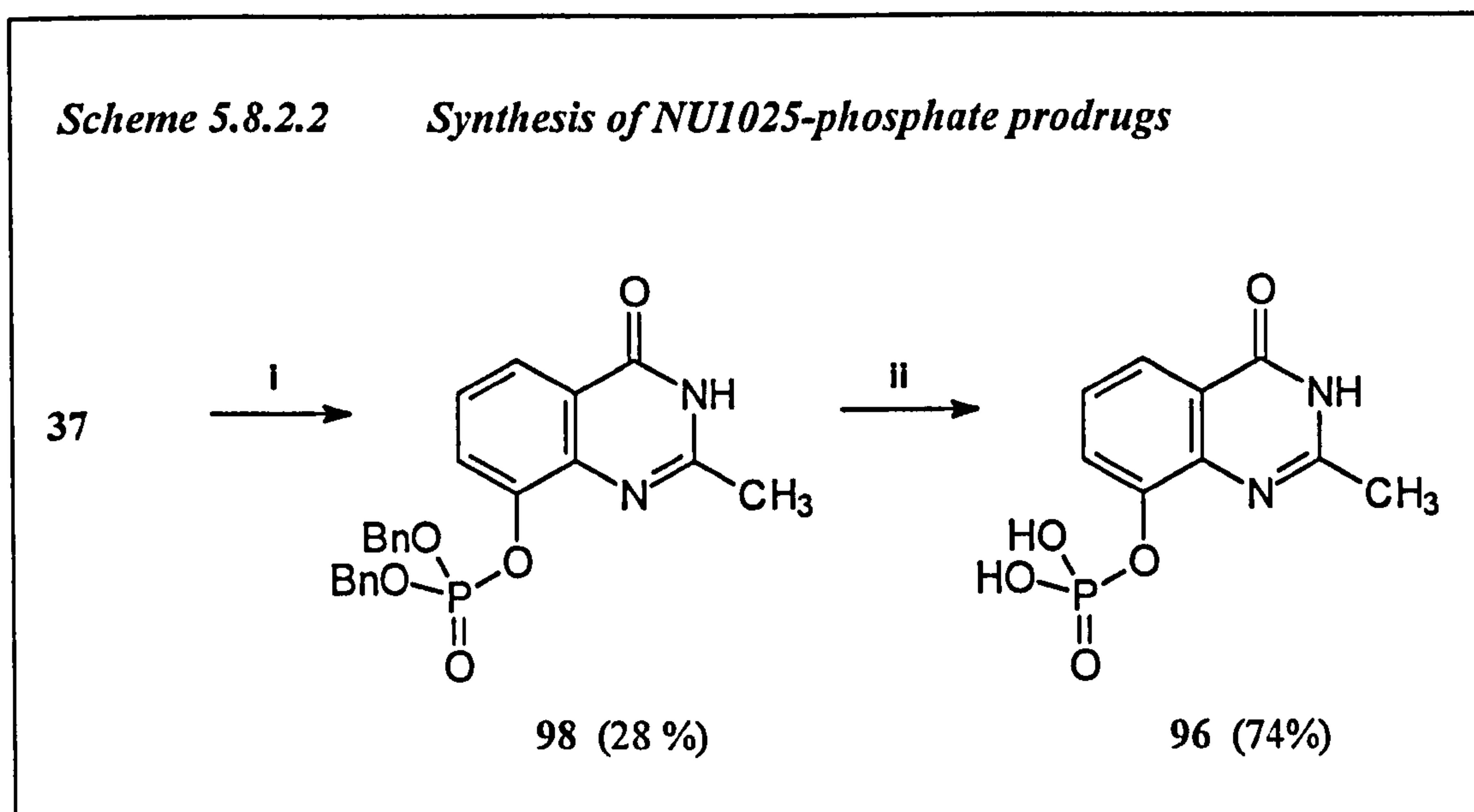
Deprotection of the diphenyl phosphate ester was attempted using a Parr hydrogenation procedure at 45-50 psi.²⁷⁹ A mixture of the diphenyl ester and platinum oxide (Adam's catalyst) in ethanol, was placed under an atmosphere of hydrogen for 4 hours, in an attempt to produce the phosphate 96. However, it was found that under these conditions, compound 37, the parent quinazolinone could be detected, suggesting that the phosphate group had been cleaved at the P-OAr bond during the hydrogenation. The product isolated gave unsatisfactory ¹H NMR data and alternative methods of phosphorylation were considered, with regard to the ease of removal of the phosphate protecting groups.

The dibenzyl phosphate ester 98 was synthesised in the expectation that the benzyl groups would be more easily removed by catalytic hydrogenation than the phenyl groups, to give the required phosphate 96. Compound 98 was synthesised in an analogous manner to 95, by treating compound 37 with dibenzyl phosphorochloridate 97, in the presence of dry EtN(ⁱPr)₂.²⁷⁹

The dibenzyl phosphorochloridate 97 was generated *in situ*, by treating dibenzyl phosphite with *N*-chlorosuccinimide.²⁸⁰ In contrast to the literature procedure, the reaction was carried out using dry acetonitrile as the solvent and not benzene. Separation of the succinimide by-product from the reaction mixture initially proved to be problematic, due to the partial solubility of succinimide in dry acetonitrile. In order to overcome this problem, three parallel reactions were carried out using dry acetonitrile, dry dichloromethane and dry ether as solvent. It was found that the by-product was least soluble in dry ether, in comparison with the other solvents used, and that the succinimide could be more easily separated from the reaction mixture. In order to quantify the amount of dibenzyl phosphorochloridate present in the reaction mixture, after removal of the succinimide by-product, aqueous ammonia was added. The resulting white precipitate was identified as dibenzyl aminophosphonate by ¹H NMR and the yield was calculated to be 65%. Subsequent preparations of dibenzyl phosphorochloridate were carried out in dry ether, and quantities were calculated according to an assumed yield of 65%. After removal of the succinimide, the dibenzyl phosphorochloridate was

redissolved in dry acetonitrile and used, as a solution, for the phosphorylation of compound **37**.

Removal of the benzyl groups of **98**, to give the free phosphate **96** was effected using a 1:1 mixture of redistilled THF (the THF was distilled from sodium/benzophenone and then redistilled from LiAlH_4)²⁸¹ and water as solvent. Hydrogenation was carried out under ambient temperature and pressure, using palladium/carbon catalyst (see *Scheme 5.8.2.2*).



Reagents : i $(\text{BnO})_2\text{P}(\text{O})\text{Cl}$, dry MeCN, dry $\text{EtN}(\text{iPr})_2$, 25 °C, ii redistilled THF:water (1:1), $\text{H}_2/\text{Pd/C}$.

A white precipitate formed on completion of the reaction, which was redissolved in an excess of water in order to remove the catalyst by filtration. The product obtained was readily soluble in aqueous sodium bicarbonate solution and was submitted for initial HPLC studies. The preliminary results were extremely promising and indicated that compound **96** is stable in aqueous solution over several days. It also appears that the conversion of **96** back to the parent quinazolinone is indeed enzyme-dependent and not pH-dependent, as observed with the carbamate ester **93**.

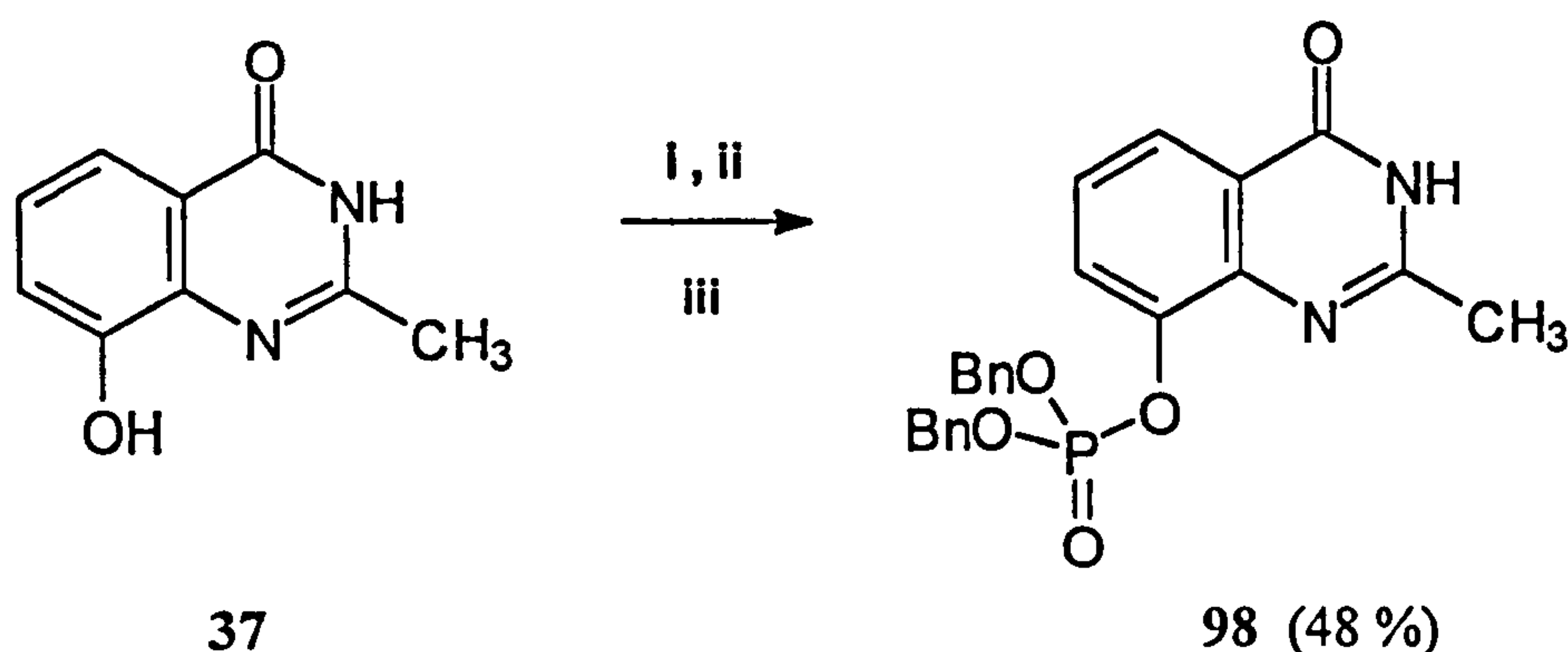
Following the success in synthesising a quinazolinone phosphate prodrug, it was necessary to produce sufficiently large quantities of the compound to facilitate *in vivo* investigations.

The formation of the dibenzyl phosphate ester **98** was found to proceed in only 28% yield. Although it is possible to recover unreacted starting material **37** and repeat the reaction, this is a time-consuming and tedious process. Another disadvantage of this reaction is the preparation of the phosphorylating species, dibenzyl phosphorochloridate **97**. The reagent is extremely unstable and decomposes readily on standing or on exposure to air/moisture.²⁸² The reagent must be freshly prepared, prior to use to minimise decomposition. Thus, reaction conditions employed here are not satisfactory for large scale synthesis of the required prodrug.

According to the literature,²⁸³ phosphorylation of amines using dibenzyl phosphorochloridate is almost quantitative. However, with alcohols, comparatively low yields are obtained. Phosphorylation of alcohols is generally carried out in the presence of a tertiary base and it has been proposed that there is a tendency for the tertiary base hydrochloride, produced during the reaction, to debenzylate the initial reaction product. Early phosphorylation reactions were carried out using pyridine as the base. However, it was found that dibenzyl phosphorochloridate is completely destroyed by pyridine in 2 hours 30 min. at 34 °C. Studies were conducted to find a less destructive base than pyridine and 2,6-lutidine was found to fulfil this requirement.²⁸³

The reaction has been carried out using the hindered base *N,N*-diisopropylethylamine. However, the yield obtained is still very low. An extensive review of the literature uncovered an alternative method for the phosphorylation of phenols.²⁸⁴ This method provides a much more convenient 'one-pot' synthesis, quoting high yields of phosphorylated phenols. *Scheme 5.8.2.3* shows the reaction pathway.

Scheme 5.8.2.3 *Alternative synthesis of 8-(O-dibenzyl phosphoryl)-2-methyl quinazolin-4-[3H]-one 98*

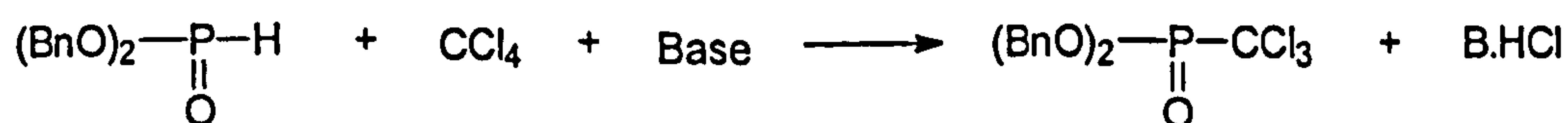


Reagents : i dry CCl_4 , dry acetonitrile, $-10\text{ }^\circ\text{C}$, ii dry $\text{EtN}(\text{iPr})_2$, DMAP, iii dibenzyl phosphite.

Care was taken to ensure that the internal temperature of the reaction mixture did not rise above $-10\text{ }^\circ\text{C}$ until the reaction was complete, as determined by TLC. Not only was the reaction convenient, mild and clean, but it was complete within 1 hour. The yield obtained when compound **37** was phosphorylated with this method was 48%, almost double the yield of the previous method used.

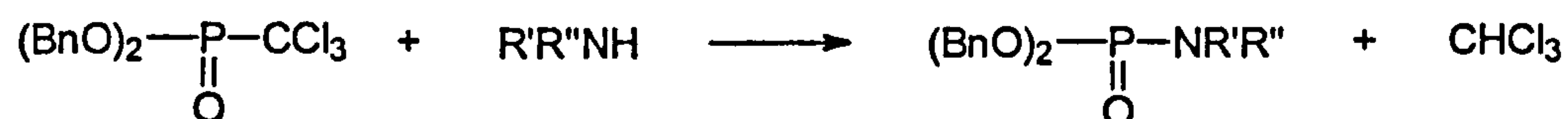
According to the literature²⁸⁵ it is assumed that the reaction occurs in two stages: initially, carbon tetrachloride reacts with dibenzyl phosphite to generate a trichloromethyl phosphonate derivative (*Eqn 1*: in this mechanism, dibenzyl phosphorochloridate is not the intermediate phosphorylating species):

Eqn 1 :



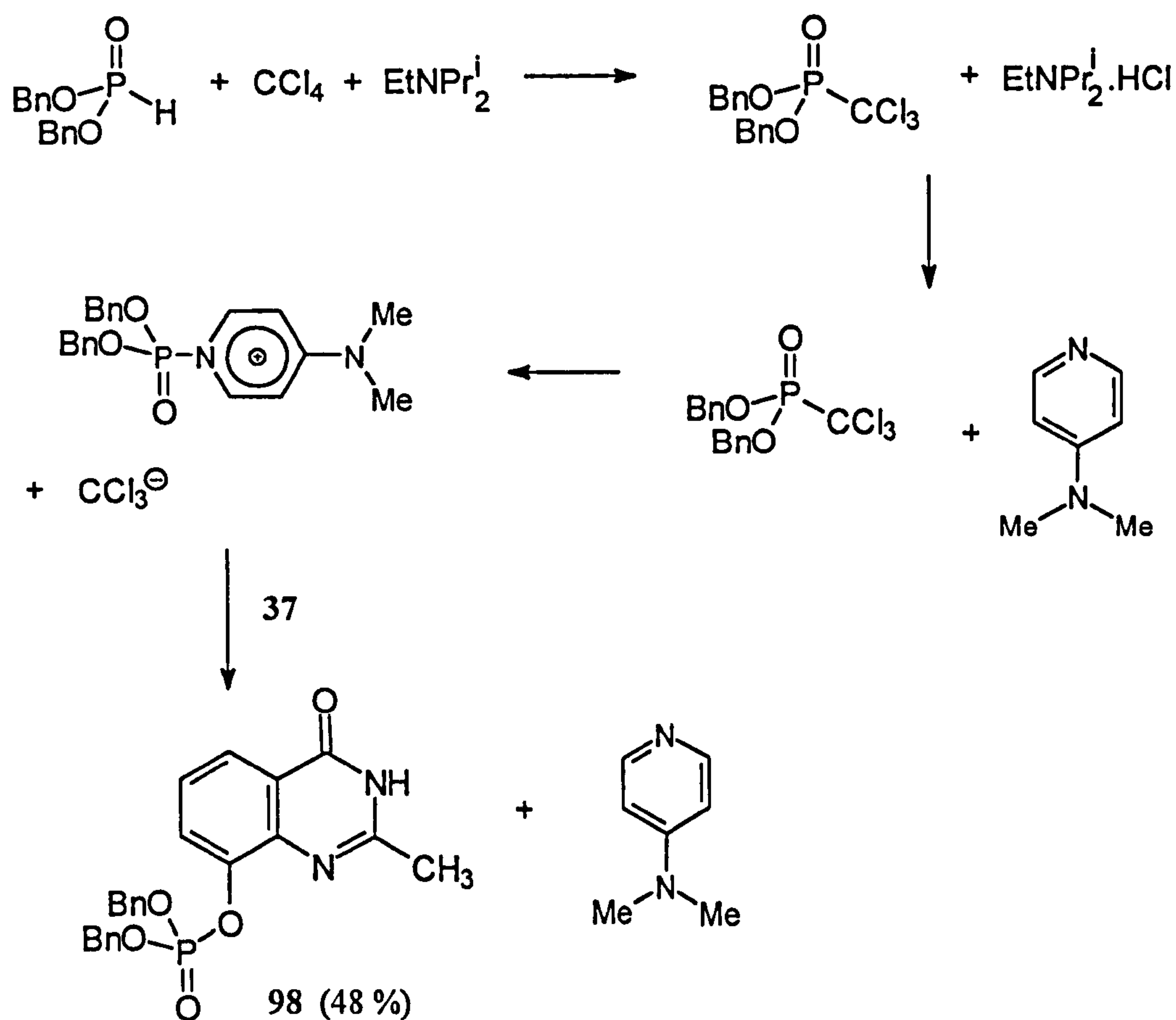
The trichloromethyl phosphonate then reacts with a molecule of primary/secondary amine, to give chloroform and an amino- or substituted aminophosphonate (*Eqn 2*) :

Eqn 2 :



The aminophosphonate can then react with a phenol to give a phosphorylated phenol and an amine. With regard to the synthesis of compound 98, the sequence of events is shown in *Scheme 5.8.2.4*.

Scheme 5.8.2.4 Mechanism of phosphorylation of NU1025 (37)



The alternative 'one-pot' phosphorylation method is convenient, mild, clean and amenable to scale-up. A sufficiently large quantity of 96 (0.65 g) was synthesised and submitted for preliminary *in vivo* investigations.

8-Methyl-2-(4'-hydroxyphenyl)quinazolin-4-[3H]-one 87 was also phosphorylated using the one-pot procedure, to give the dibenzyl phosphate ester 99, in 26% yield. Unfortunately, time constraints did not allow the deprotection of this compound to give the free phosphate.

5.9 Summary

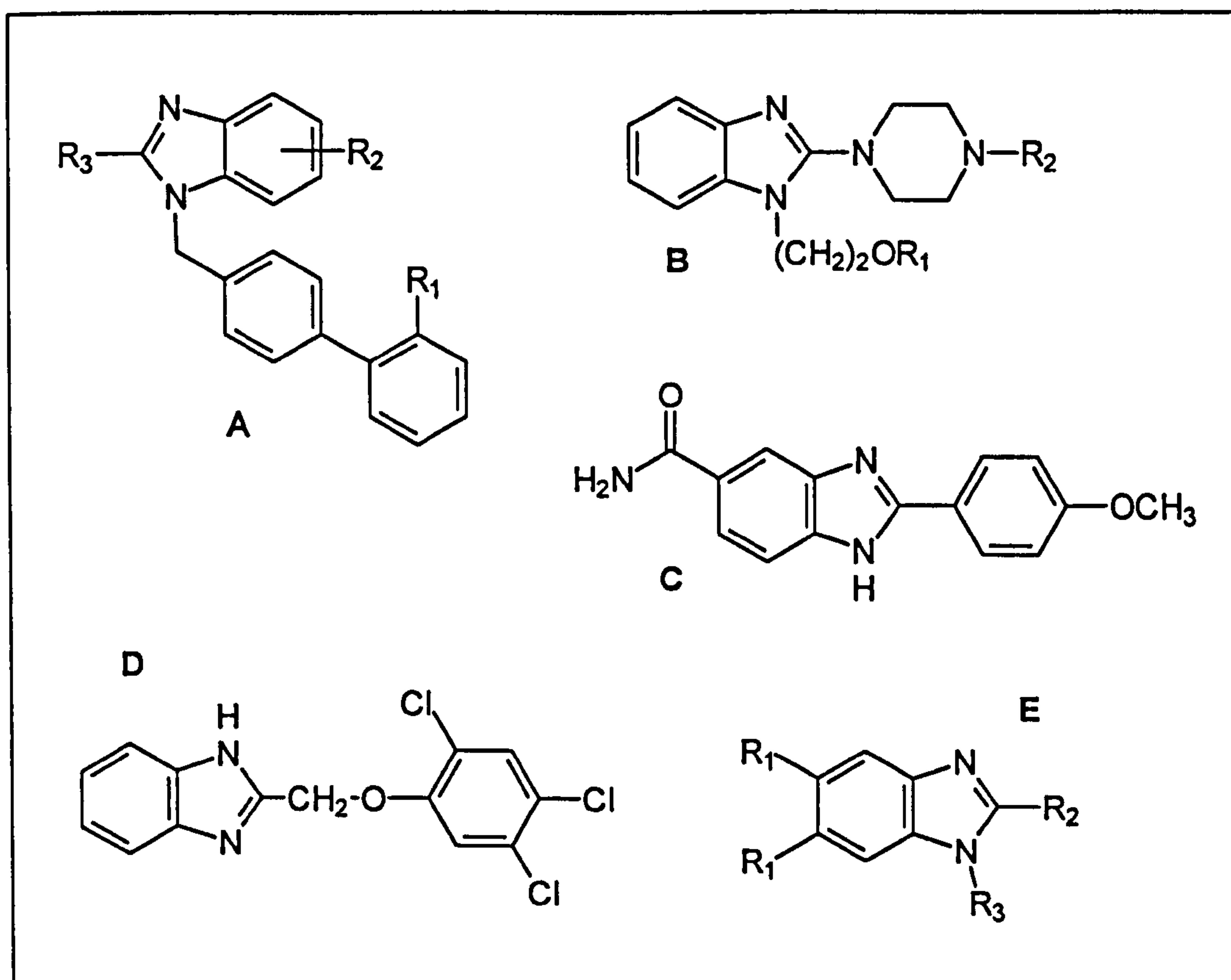
An efficient high-yielding synthetic pathway has been developed for the quinazolin-4-[3H]-ones. The scheme works effectively in practice and has led to the synthesis of a large series of compounds exhibiting potent PARP inhibitory activity. Limitations of the synthetic route are, however, apparent and modifications to the scheme should be considered. A mild, rapid phosphorylation procedure has been used to prepare a water-soluble prodrug derivative of NU1025 (37). The prodrug 8-(O-phosphoryl)-2-methylquinazolin-4-[3H]-one 96, has been submitted for preliminary *in vivo* studies.

Chapter Six: Development of the Benzimidazole Series

6.1 Benzimidazoles as Pharmaceutical Agents

Benzimidazole derivatives are known to possess a wide range of biological activities, including antibacterial, antifungal and anthelmintic.²⁸⁶ *Figure 6.1* illustrates some examples of commercially significant benzimidazole derivatives.

Figure 6.1 Examples of Biologically Active Benzimidazoles



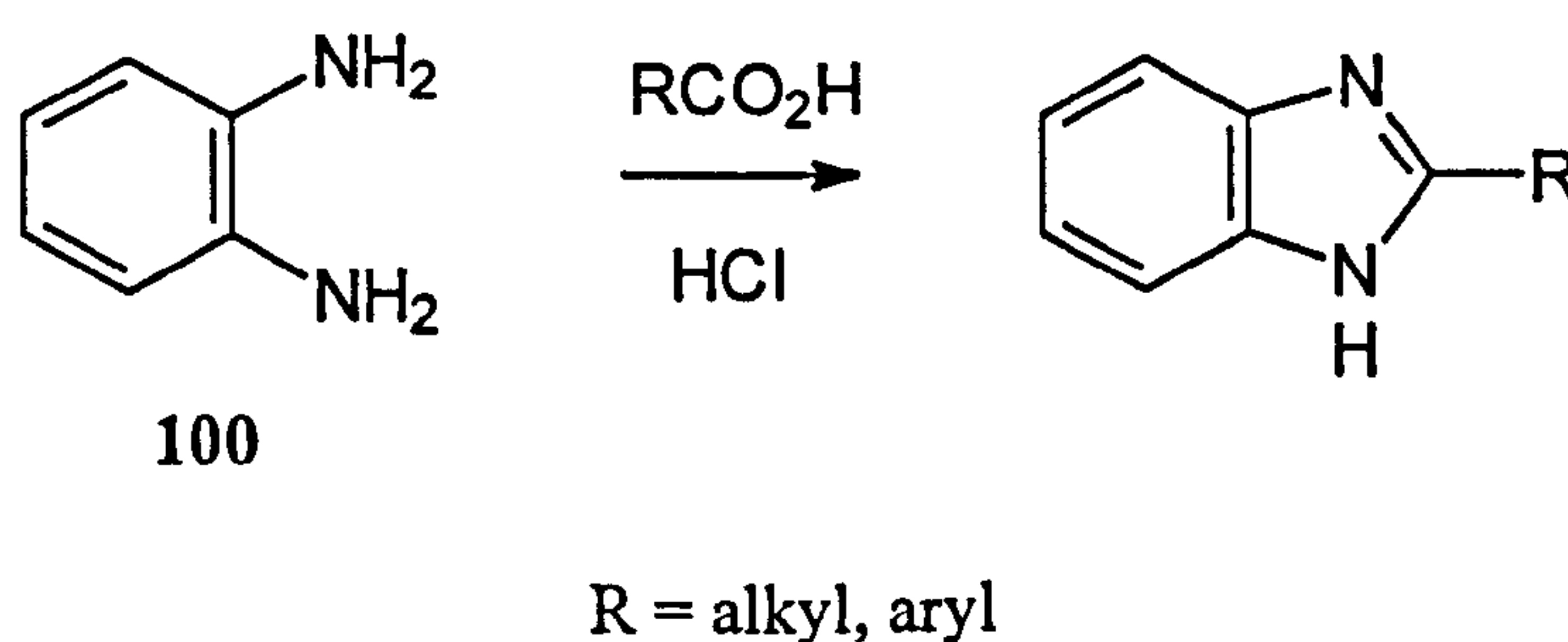
Compounds of general structure A have been shown to interfere with the renin-angiotensin system and are effective in the treatment of hypertension and congestive heart failure.^{287,288} Currently used clinical agents include the angiotensin converting enzyme (ACE) inhibitors captopril and enalapril.²⁸⁸ However, it is hoped that non-

peptide angiotensin II receptor antagonists of type A will be longer acting when administered orally.^{287,288} Compounds of structure B are 2-(4-substituted-1-piperazinyl) benzimidazoles and exhibit potent H₁-antihistaminic activity both *in vitro* and *in vivo*.²⁸⁹ Compound C has been identified as a novel topoisomerase I poison.²⁹⁰ Relatively few topoisomerase I poisons are known but they represent a class of pharmacological agents with interesting antineoplastic activity.²⁹⁰ Compound D exhibits growth-stimulating activity on the roots and cotyledons of *Zea mays*²⁹¹ and compounds of general structure E have been shown to possess antimicrobial activities against different strains of bacteria and fungi.²⁹²

6.2 General Methods of Benzimidazole Synthesis

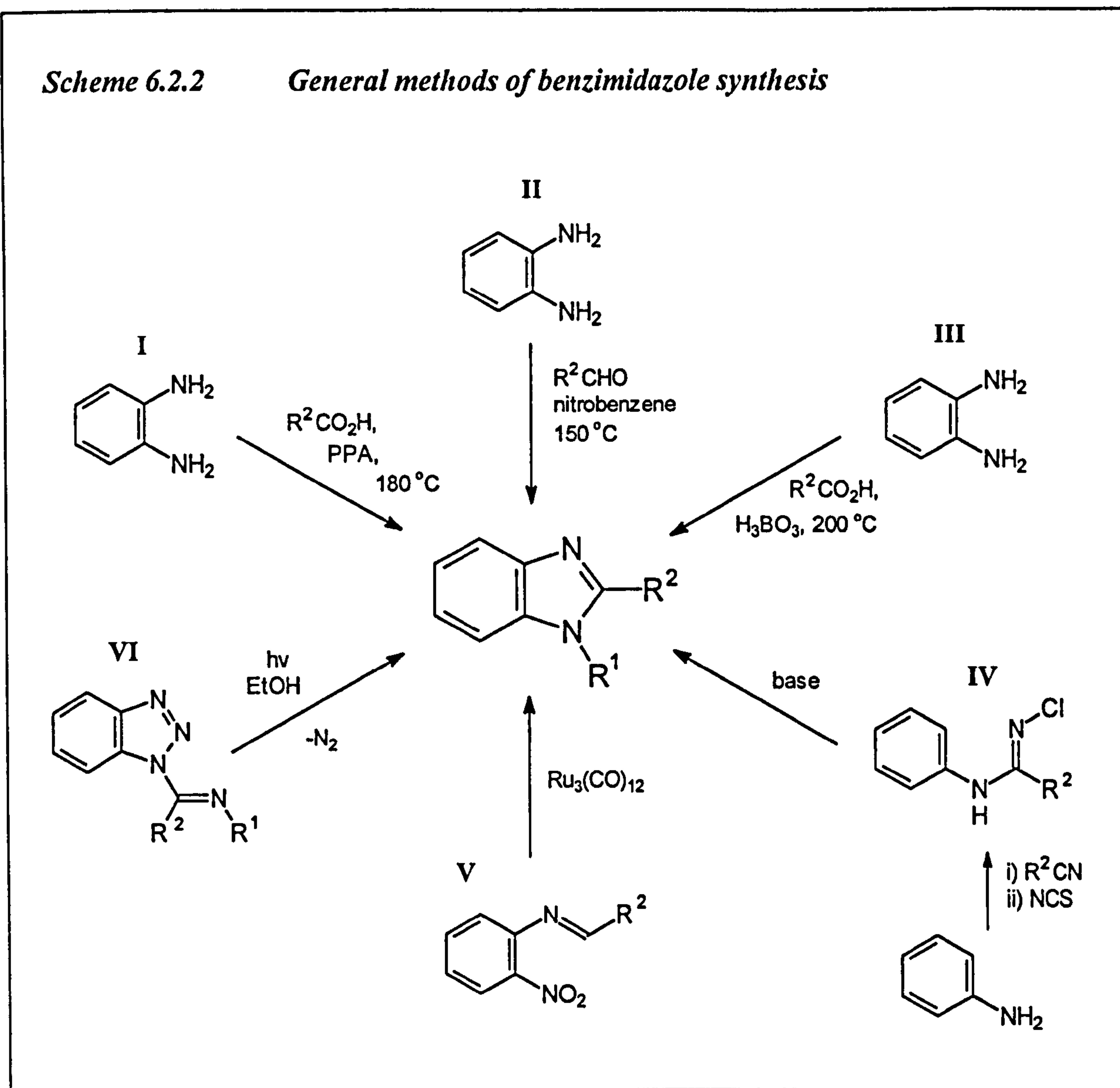
The conventional method of benzimidazole synthesis, known as the method of Phillips, involves the reaction of 2-phenylenediamine 100 with the appropriate carboxylic acid, in the presence of a mineral acid catalyst (see *Scheme 6.2.1*).^{293,294}

Scheme 6.2.1 *The Phillips method of benzimidazole synthesis*^{293,294}



Variations of the Phillips method have been investigated in order to improve yields. For example, the use of polyphosphoric acid (PPA) as catalyst and solvent (*Scheme 6.2.2*, method I) usually gives higher yields of benzimidazoles.²⁹⁵ [For methods I-V, R¹ = H].

Scheme 6.2.2 General methods of benzimidazole synthesis



Method II was used for the preparation of potential topoisomerase I poisons, similar to compound **C** (Figure 6.1).²⁹⁰ Method III, a variation of the Phillips method, was used for the preparation of 2-substituted benzimidazoles which were investigated for growth-regulating activity.²⁹¹ Method IV illustrates a method of benzimidazole synthesis from an amidine intermediate, which is formed through the reaction of aniline with an aryl nitrile and *N*-chlorosuccinimide (NCS).²⁵¹ Method V is an example of transition-metal catalysed formation of 2-substituted benzimidazoles from 2-substituted nitrobenzenes²⁹⁶ and method VI shows a novel synthesis of 1,2-disubstituted benzimidazoles, formed by the photolysis of imidoylbenzotriazoles.²⁹⁷

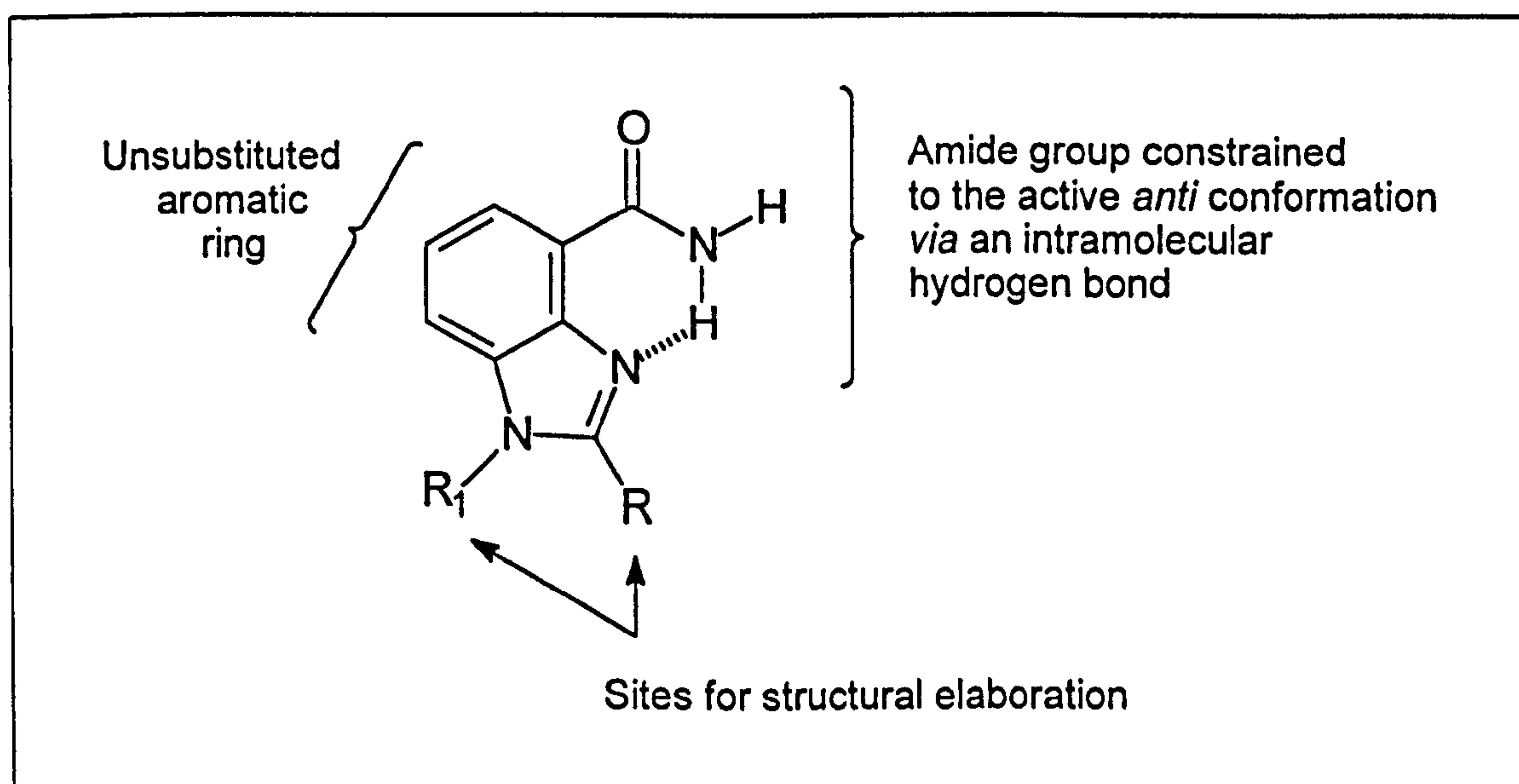
6.3 Benzimidazoles as PARP Inhibitors

As part of the ongoing research at Newcastle University, a novel series of benzimidazole-4-carboxamides, exhibiting extremely potent PARP inhibitory activity, was developed.²⁵¹ Following the synthesis and biological evaluation of the benzoxazole-4-carboxamides,²⁶³ the benzimidazole-4-carboxamides were examined as isosteric counterparts. The carboxamide group is constrained *via* an intramolecular hydrogen bond, in an analogous manner to the benzoxazoles but the presence of the N^1 imidazole nitrogen allows an additional site on the compounds to be elaborated.

6.3.1 Benzimidazole Structure-Activity Relationships

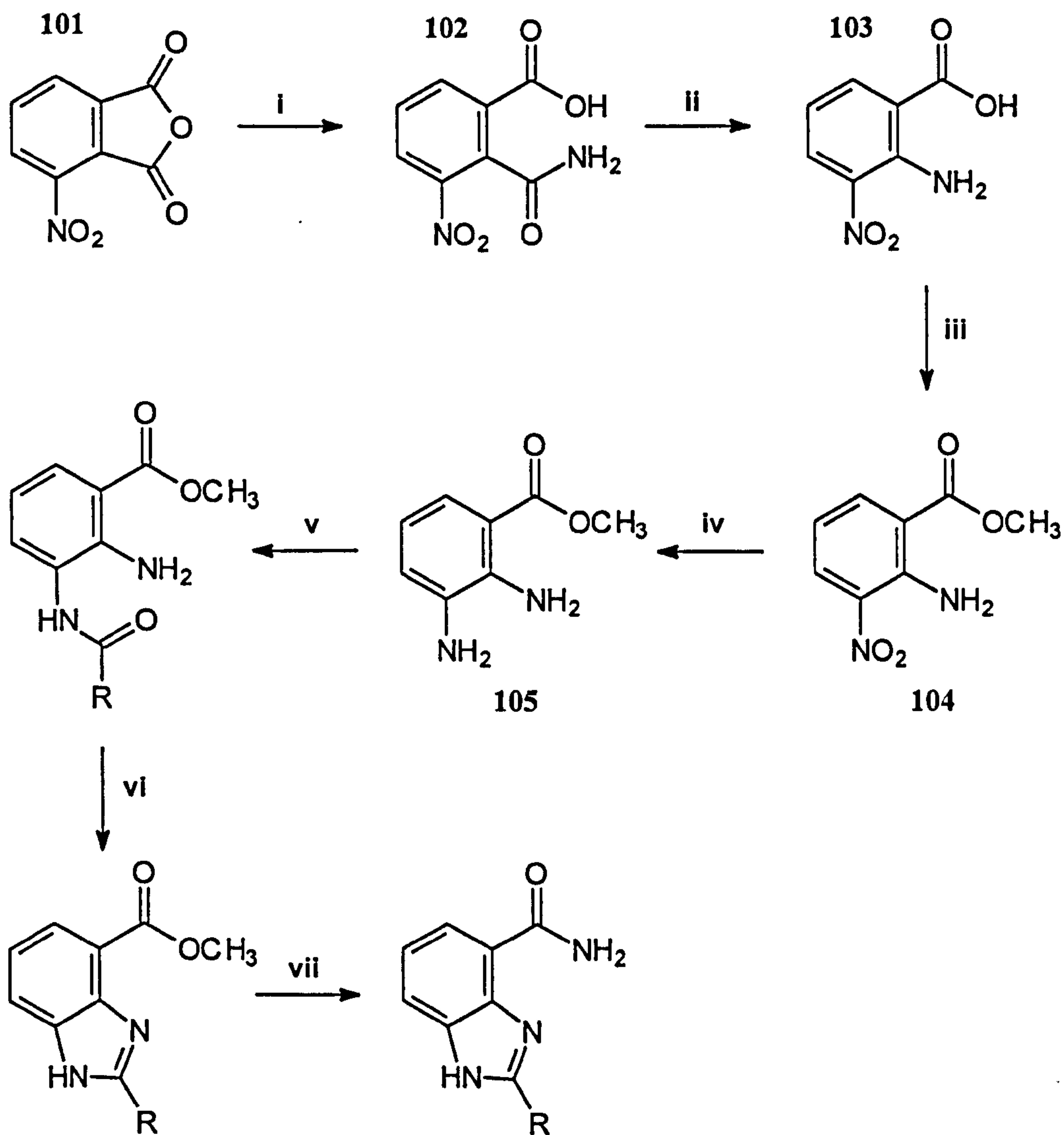
The benzimidazoles are similar in electronic configuration to the benzoxazoles and possess the structure-activity requirements previously determined for optimal PARP inhibition (see section 4.3). *Figure 6.3.1* shows the structural features of the benzimidazole-4-carboxamides.²⁵¹

*Figure 6.3.1 Benzimidazole structure-activity relationships*²⁵¹



6.3.2 Proposed Synthetic Route to Benzimidazole-4-Carboxamides

Scheme 6.3.2 Synthesis of benzimidazole-4-carboxamides



Reagents : i NH_4OH , ii KOH , Br_2 , H_2O , $60\text{ }^\circ\text{C}$, 3h, iii HCl (g) , MeOH , iv $\text{H}_2/\text{Pd/C}$, MeOH , v RCOCl , NEt_3 , DMAP , dry THF , $-10\text{ }^\circ\text{C}$, vi $\text{CH}_3\text{CO}_2\text{H}$, $120\text{ }^\circ\text{C}$, vii $\text{NH}_3\text{ (l)}$, $80\text{ }^\circ\text{C}$, 40 atm.

The synthetic route developed for the synthesis of benzimidazole-4-carboxamides is shown in *Scheme 6.3.2*.²⁵¹ Starting with commercially available 3-nitrophthalic anhydride **101**, reaction with aq. ammonia gave 3-nitrophthalamic acid **102**, which underwent Hofmann degradation to give 3-nitroanthranilic acid (3-nitro-2-aminobenzoic acid) **103**. Conversion into the methyl ester **104** was achieved using HCl gas and methanol. The resulting ester was catalytically reduced to give methyl 2,3-diaminobenzoate **105** which was selectively acylated with a range of acid chlorides at reduced temperature. The *N*-acyl derivatives were cyclised using glacial acetic acid to give the methyl benzimidazole-4-carboxylates. These were converted to the target benzimidazoles by high pressure reaction with ammonia.

A series of 2-substituted benzimidazole derivatives was synthesised by this route and the 2-substituents included alkyl, aryl and substituted-aryl functionalities. The benzimidazoles were found to be significantly more potent than the corresponding benzoxazoles. Although no clear structure-activity relationships were established for the benzimidazole-4-carboxamides, they are among the most potent PARP inhibitors known to date.²⁵¹ Compounds bearing electron-withdrawing and electron-donating groups in the 4'-position were both found to exhibit excellent PARP inhibitory activity in the nanomolar concentration range (see *Figure 6.3.2*).²⁵¹

It was found that substitution at the 3'-position did not significantly alter inhibitory activity, as indicated by compounds **111** and **112**. A second series of compounds bearing substituents on the *N*¹ imidazole nitrogen was synthesised. Introduction of a methyl group at the *N*¹-position was tolerated (**114**) but any further increase in steric bulk resulted in a decrease in activity (**115** and **116**). The 2-alkyl benzimidazole, 2-methyl-1*H*-benzimidazole-4-carboxamide (NU1064) was selected for use in potentiation studies and was shown to enhance the cytotoxicity of the alkylating agent temozolomide.²⁵¹ Data from kinetic experiments indicated that NU1064 acts as a competitive inhibitor.²⁵¹

Figure 6.3.2 Benzimidazole PARP Inhibitors^{251*}

	R ₁	R ₂	R ₃	IC ₅₀ (nM)
106	NO ₂	H	H	19
107	CN	H	H	27
108	OCH ₃	H	H	59
109	OH	H	H	77
110	CF ₃	H	H	75
111	H	CF ₃	H	76
112	H	OCH ₃	H	130
113	OCH ₃	H	H	59
114	OCH ₃	H	CH ₃	68
115	OCH ₃	H	PhCO	270
116	OCH ₃	H	BnO ₂ C	1940

It was deemed necessary to continue the expansion of the benzimidazole series, as these derivatives possess excellent PARP inhibitory activity, and further quantities of benzimidazole-4-carboxamides were required for use in potentiation studies.

6.4 Aims of Benzimidazole Research

A benzimidazole prodrug, analogous to the quinazolinone prodrug (Chapter Five, Section 5.8.2) was previously synthesised in low yield, from 2-(4'-hydroxyphenyl)-1*H*-benzimidazole-4-carboxamide 109.²⁵¹ It was necessary to synthesise bulk quantities of 109 for subsequent prodrug modification and this was initially attempted using the original synthetic pathway (*Scheme 6.3.2*). It was hoped that this pathway would not only allow scale-up preparations to be carried out but also the synthesis of 2', 3'-disubstituted benzimidazoles, to expand the existing series of compounds.

*See Chapter Seven, Section 7.2.1 for assay details

6.5 Syntheses of Benzimidazole PARP Inhibitors

Compound **103** was prepared in bulk[§] for the subsequent preparation of 2-(4'-hydroxyphenyl)-1*H*-benzimidazole-4-carboxamide **109**. The derivative was synthesised in good yield and stored in a dark glass bottle until needed. Conversion of **103** to the corresponding methyl ester, methyl 2-amino-3-nitrobenzoate **104**, was performed in order to improve the solubility characteristics of the molecule.²⁵¹ The ester **104** was obtained by saturating a solution of **103** in methanol at -10 °C, with dry HCl gas. The saturated solution was allowed to warm up to room temperature and heated under reflux under dry conditions, for six hours. After cooling and removal of the resulting bright yellow precipitate by filtration, the mother liquor was found by TLC to contain only product and unreacted starting material. These compounds were separated by column chromatography and a corrected yield of 86% was recorded for compound **104** (lit. yield²⁵¹ 60%).

Small scale reactions using the pathway in *Scheme 6.3.2* initially proved successful.²⁵¹ However, subsequent attempts to reproduce comparable yields under the same conditions were unsuccessful.

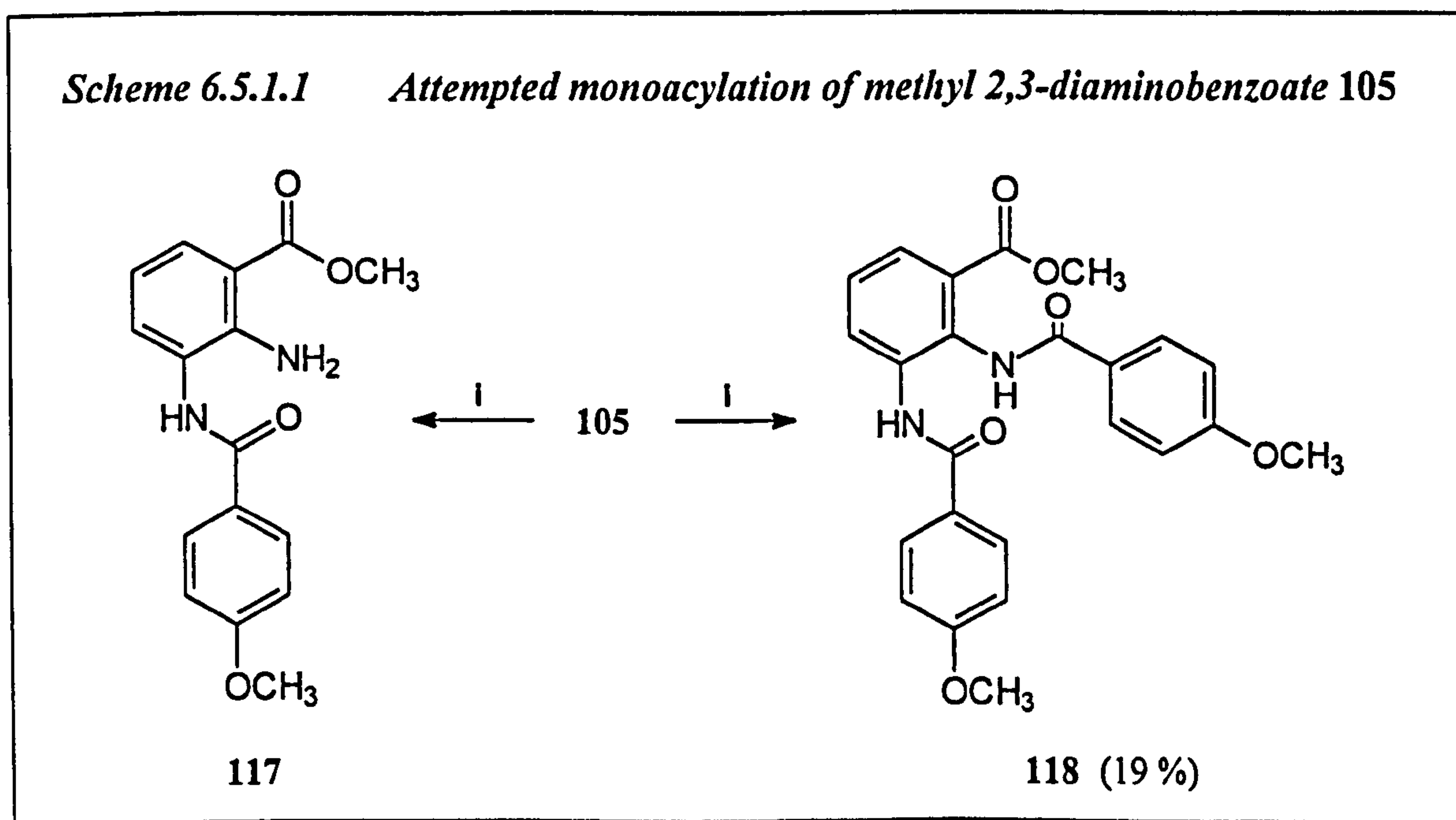
6.5.1 Limitations of the Proposed Synthetic Route

Methyl 2,3-diaminobenzoate **105** is known to be extremely light-sensitive and readily decomposes on exposure to light.²⁵¹ For this reason, it was proposed that **105** should be freshly prepared, prior to *N*-acylation. The ester **105** was prepared by catalytic hydrogenation of **104**, using 10% Pd/C catalyst and methanol as solvent.²⁵¹ Once hydrogen absorption was complete, the resulting colourless solution was rapidly seen to colour during the subsequent handling and isolation of the compound, the end result being the formation of a dark brown tar-like substance. Immediate ¹H NMR analysis of the crude product showed the required compound to be present.

[§] Prepared by Mr R. Davison

However, difficulties were encountered in the calculation of yields, due mainly to the presence of breakdown products. Attempts to carry out the reaction work up with minimal exposure of the compound to light and air did not help, and attention was directed towards improving the handling properties of compound **105** (see Section 6.5.2).

Preparation of the *N*-acyl derivative, methyl 2-amino-3-*N*-(4'-methoxybenzoyl) aminobenzoate **117** was previously achieved using 4-methoxybenzoyl chloride in the presence of dry NEt₃ and DMAP in cold, dry THF.²⁵¹ The compound was prepared in 62% yield.²⁵¹ It was reasoned on the basis of electronic and steric factors, that the 3-amino group should be more nucleophilic than the 2-amino group and a single acylation at the 3-position should occur. However, when acylation of crude **105** was attempted using the above reaction conditions, multiple products were detected by TLC, both under short wave and long wave light. TLC comparisons with authentic material indicated that the required monoacylated product was present, albeit in low yield. The major product, isolated in 19% yield, was found to be the diacylated compound **118** (see *Scheme 6.5.1.1*).



Reagents : i dry THF, *p*-CH₃C₆H₄COCl, dry NEt₃, DMAP (5 mol. %), -10 °C.

Hence, it was found that under these conditions, the slightly enhanced nucleophilicity of the 3-amino group did not result in the selective mono-acylation of **105**.

The last two synthetic steps were found to be non-problematic. Cyclisation of the mono-acylated derivative **117** in glacial acetic acid at 120 °C resulted in the formation of the ring-closed methyl benzimidazole-4-carboxylate, which was isolated as its acetate salt **120** in 74% yield (lit. yield²⁵¹ 75%). High pressure reaction of **120** with freshly condensed, liquid ammonia gave 2-(4'-methoxyphenyl)-1*H*-benzimidazole-4-carboxamide **121** in 87% yield (lit. yield²⁵¹ 80%). Compound **121** is the direct precursor of 2-(4'-hydroxyphenyl)-1*H*-benzimidazole-4-carboxamide **109**. Demethylation of **121** was carried out using BBr₃/DCM in an analogous manner to quinazolinone demethylation. The required phenolic benzimidazole **109** was isolated in 44% yield (lit. yield²⁵¹ 57%).

Following the original synthetic route, preparation of **109** was achieved in 21% overall yield, with respect to **105**.²⁵¹ Repetition of the synthesis on a slightly larger scale led to ineffective *N*-acylation, due to the lack of selectivity between the two amino groups of **105**. This resulted in a reduced overall yield of only 7% for **109**, with respect to **105**. Clearly, the route employed is not high-yielding, reproducible or amenable to scale-up. For the purpose of producing larger quantities of **109**, it was necessary to introduce modifications to the original synthetic route.

6.5.2 Modifications to the Proposed Synthetic Route

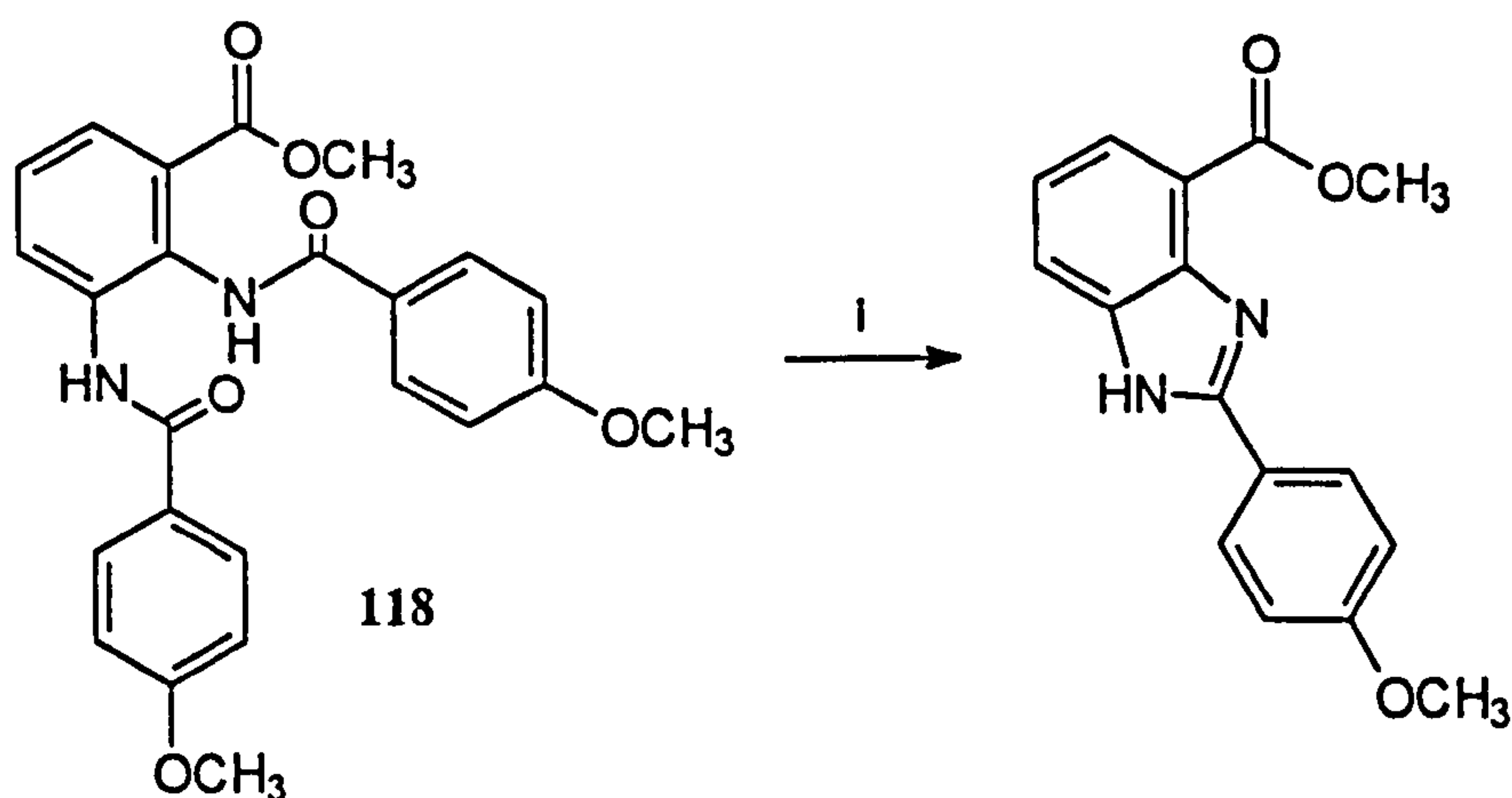
It appears that the two major problems involved are the instability of compound **105** and the non-selectivity of the acylation step. In an attempt to improve the handling characteristics of **105**, it was proposed that the derivative could be isolated as a hydrochloride salt. Methyl 2-nitro-3-aminobenzoate **104** was dissolved in cold, dry methanol. Two equivalents of acetyl chloride were added in order to generate HCl *in situ* and the reaction mixture was hydrogenated in the presence of 10% Pd/C catalyst. The resulting colourless solution was treated as light-sensitive and care was taken to minimise

exposure to light and air. The product was identified as the monohydrochloride salt **119** and was isolated as a pink solid in 88% yield. The salt proved to be more easily transferable than the previously obtained brown oil. Although some discolourisation was evident, the reaction was considered an improvement on the original procedure. Despite the stability of **105** being improved by isolating the compound as a salt, this did not lead to any significant difference in the yield of **117** (25% yield from **119**). Selectivity between the amino groups was still found to be a problem and it was decided to tackle this step with a different approach.

6.5.3 Synthesis of Benzimidazoles from Diacylated Precursors

Since the formation of diacylated compounds appeared unavoidable, it was proposed that benzimidazole synthesis could be attempted from diacylated precursors. A recent review in the literature details the *p*-toluenesulfonic acid-promoted synthesis of 2-substituted benzoxazoles and benzimidazoles from diacylated precursors, and quotes excellent yields for a range of compounds containing electron-withdrawing or electron-donating substituents in the 2-position.²⁹⁸

Scheme 6.5.3 *Synthesis of benzimidazoles from diacylated precursors*



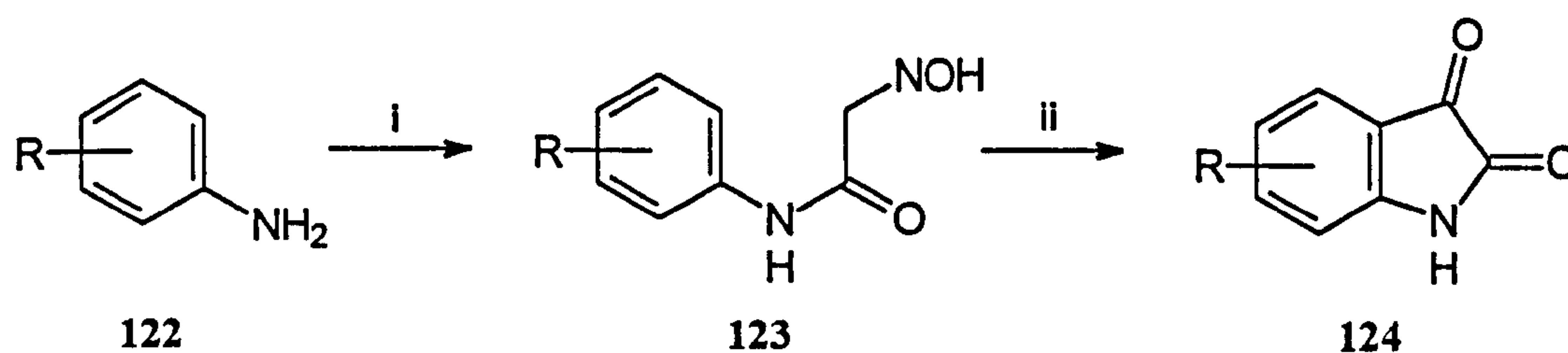
Reagents : i *p*-TsOH, xylene, 140 °C.

An analogous reaction was attempted with compound **118**. Although multiple products were detected by TLC, the major product appeared to be the required methyl benzimidazole-4-carboxylate. However, in view of the poor yields of **118** obtained, it was deemed inappropriate to use this compound as a starting material.

6.5.4 Synthesis of Benzimidazoles from Isatin Derivatives

In order to address the selectivity problem of the acylation step, it was proposed that benzimidazole formation could be achieved from isatin derivatives. These have been employed as important synthetic intermediates in both the pharmaceutical and dye industries for many years.²⁹⁹ The most frequently used method of synthesis for isatin is the Sandmeyer procedure (*Scheme 6.5.4.1*).³⁰⁰

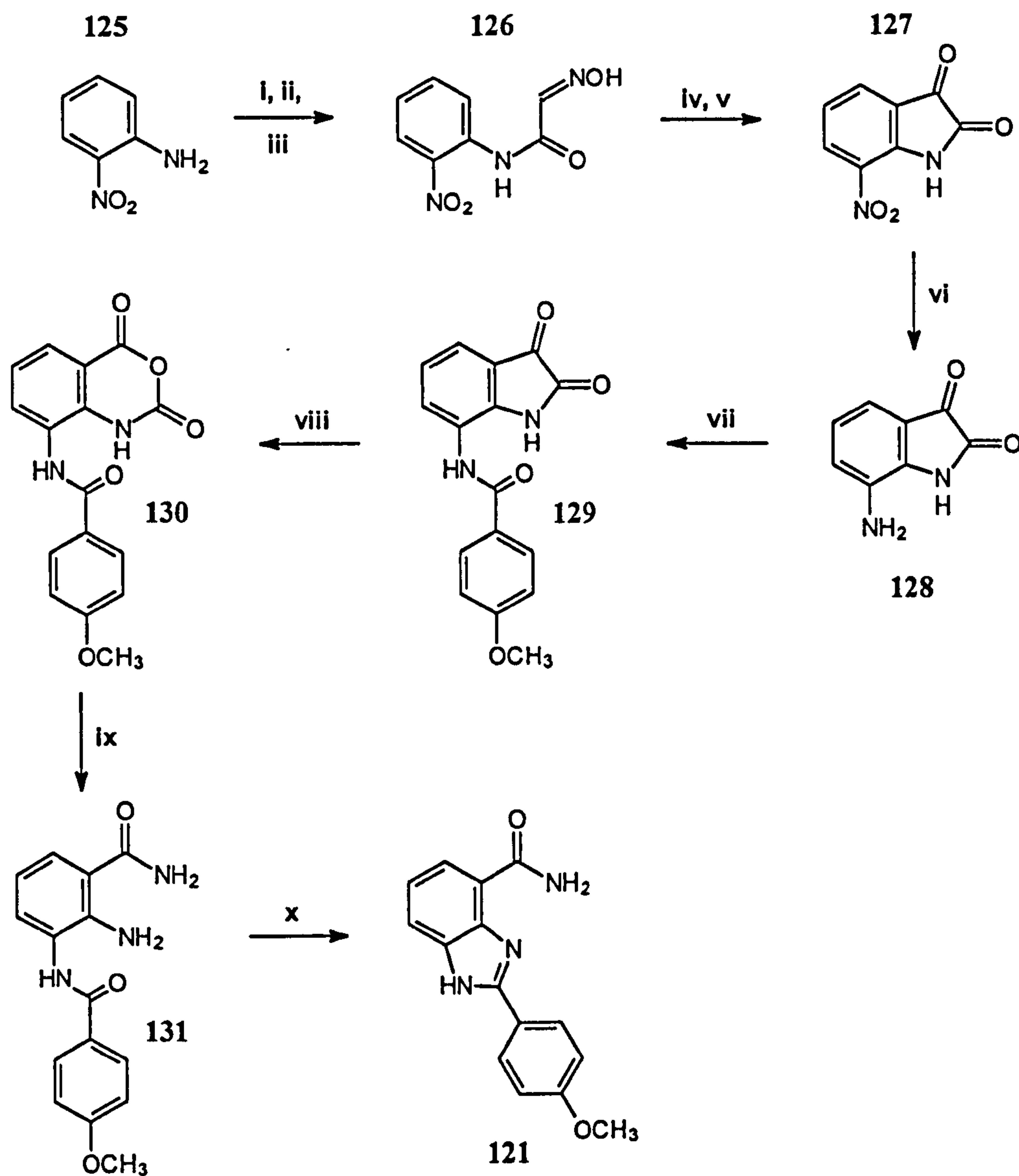
Scheme 6.5.4.1 Synthesis of isatins using the Sandmeyer procedure



Reagents : i CCl_3CHO , hydroxylamine hydrochloride, ii H_2SO_4 (conc.)

The procedure involves the formation of an isonitrosoacetanilide **123** from an aniline **122** chloral hydrate and hydroxylamine. The isonitrosoacetanilide **123** can then be converted to an isatin **124** by treatment with concentrated sulfuric acid. *Scheme 6.5.4.2* outlines the proposed route to benzimidazoles from isatin derivatives. The amino group at the corresponding 2-position of **103** is effectively restricted along with the carbonyl function into a ring system. 7-Aminoisatin **128** could then be monoacylated to give **129**.

Scheme 6.5.4.2 Synthesis of benzimidazoles from isatin derivatives



Reagents : i Na_2SO_4 , H_2O , CCl_3CHO , 40°C , ii HCl , iii hydroxylamine hydrochloride, H_2O , reflux, iv conc. H_2SO_4 , v H_2O (s), vi $\text{H}_2/\text{Pd/C}$, MeOH , vii 4- $\text{OCH}_3\text{C}_6\text{H}_4\text{COCl}$, dry NEt_3 , DMAP (5 mol. %), dry THF , -10°C , viii *m*CPBA, DCM , ix NH_4OH , x $\text{CH}_3\text{CO}_2\text{H}$, 120°C .

without interference from other functional groups. It is well known that isatin derivatives can be oxidised to give isatoic anhydrides.²⁹⁹ Subsequent treatment of the isatoic

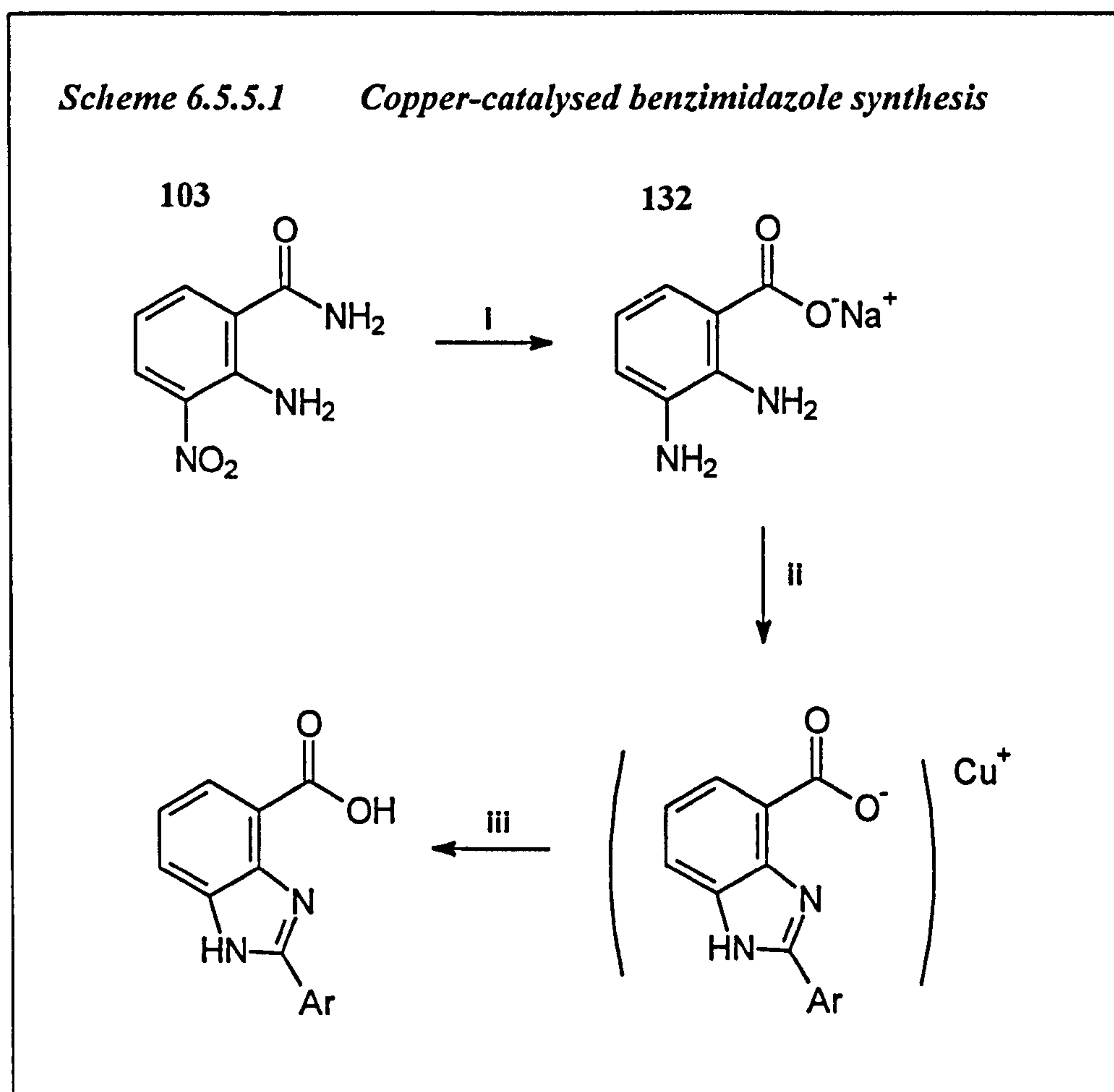
anhydride 130 with ammonia would result in nucleophilic attack of the carboxylate carbon, followed by elimination of carbon dioxide to give 2-amino-3-*N*-(4'-methoxybenzoyl) aminobenzamide 131, which could then be cyclised as before, to give the target benzimidazole 121.

The synthesis of 7-nitroisatin 127 was reported by Buchman *et al* in 1947.³⁰¹ However, no subsequent reports on the preparation of this compound have appeared since. Following the method of Buchman *et al*, based on the Sandmeyer procedure, the synthesis of isonitrosoacet-*o*-nitroanilide 126 was attempted from commercially available *o*-nitroaniline 125. Anhydrous sodium sulphate and chloral hydrate were heated to 40 °C in water to effect complete dissolution. A mixture of *o*-nitroaniline and concentrated hydrochloric acid in water was added and the reaction mixture was vigorously stirred. A solution of hydroxylamine hydrochloride in water was added and the reaction mixture was rapidly heated to boiling and maintained at boiling point for fifteen minutes. On cooling, a bright yellow precipitate formed, which was collected by filtration. The crude product was formed in 32% yield and was identified by ¹H NMR analysis to be the required product 126.

As was previously noted with the synthesis of isatin, a considerable amount of tarry material is formed during heating if the aniline derivative is not in solution.³⁰² This problem was encountered during the synthesis of 126, due to the insolubility of *o*-nitroaniline 125. Careful heating of 125 in water containing concentrated HCl gave a clear orange solution and served to eliminate the majority of the tarry residue. However, there was no significant improvement in the yield of 126. The presence of the strongly electron-withdrawing nitro group would severely reduce the nucleophilicity of the amino group nitrogen and subsequent reaction at this nitrogen would not readily occur. Repeated attempts to improve upon the yields of 126 were unsuccessful and since 7-nitroisatin was required as a starting material, it was decided to abandon the synthesis of benzimidazoles from isatin derivatives.

6.5.5 Copper-catalysed Benzimidazole Synthesis

The synthesis of 2-phenylbenzimidazole-4-carboxamides has been reported by Denny *et al.*³⁰³ The benzimidazoles were synthesised according to *Scheme 6.5.5.1*, via the oxidative condensation of 1,2-diaminobenzoic acids and aldehydes with cupric ion.³⁰⁴

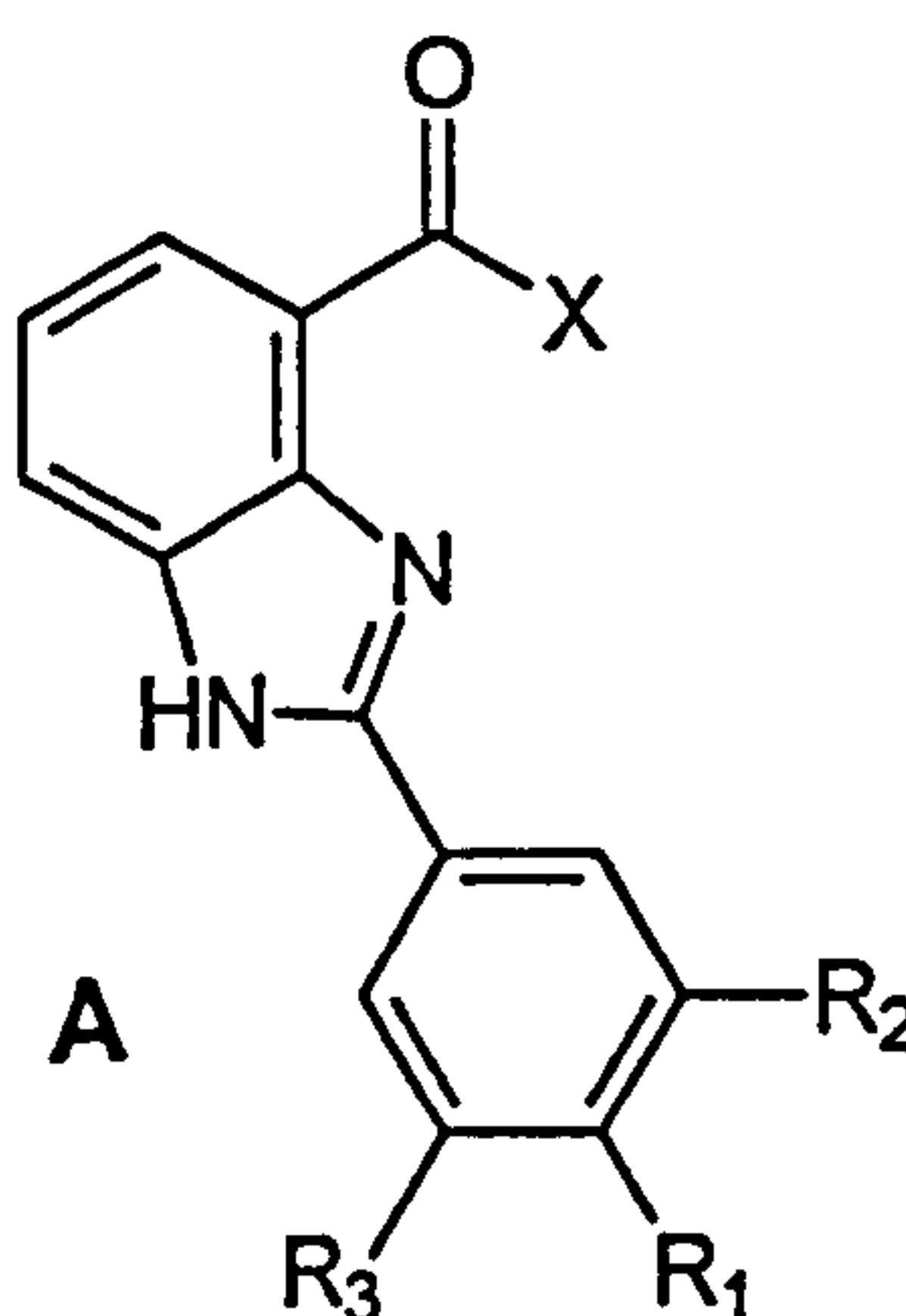
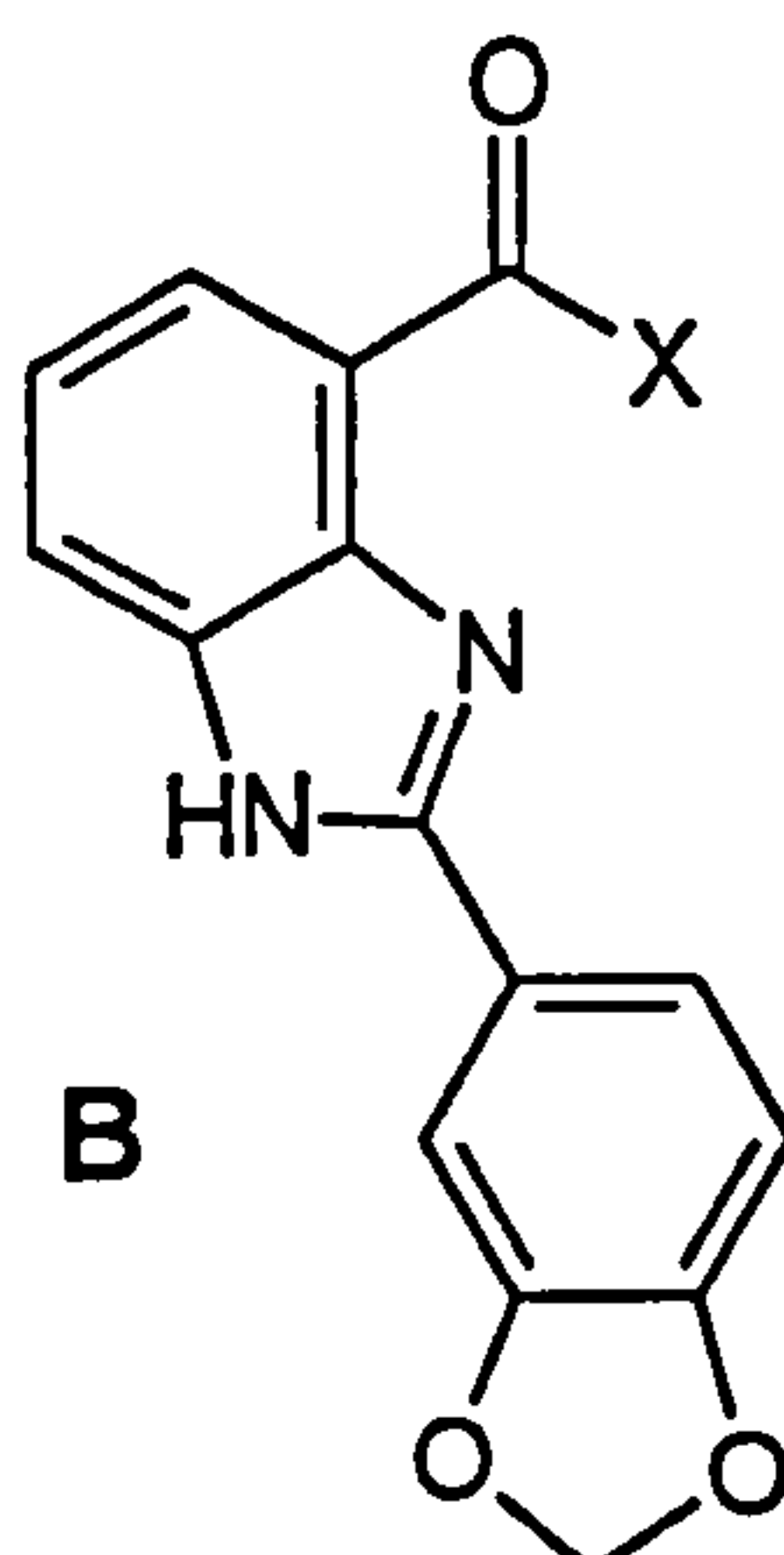


Reagents : i NaOH, H₂O, H₂/Pd/C, ii ArCHO, Cu(OAc)₂, iii Na₂S, HCl.

The advantage of this method is that the light-sensitive 2,3-diaminobenzoic acid 133 is generated *in situ* and reacted further without isolation. The insoluble benzimidazole-copper complexes can easily be separated by filtration, and the copper is removed by treatment with sodium sulfide in acidic media.

Using the above method, a series of substituted benzimidazoles were synthesised. Conversion of the benzimidazoles to the target carboxamides was achieved by treating the carboxylic acids with thionyl chloride, DMF and aqueous ammonia. *Figure 6.5.5* shows the compounds synthesised by this method.

Figure 6.5.5 Benzimidazole Derivatives

	Structure	No.	X	R ₁	R ₂	R ₃	Yield
	A	133	OH	Cl	H	H	80%
	A	134	NH ₂	Cl	H	H	47%
	A	135	OH	OH	H	H	17%
	A	109	NH ₂	OH	H	H	75%
	A	136	OH	H	OCH ₃	H	50%
	A	137	NH ₂	H	OCH ₃	H	75%
	A	138	OH	H	OH	H	67%
	A	139	NH ₂	H	OH	H	92%
	A	140	OH	OH	OCH ₃	H	44%
	A	141	NH ₂	OH	OCH ₃	H	46%
	A	142	OH	OCH ₃	OCH ₃	H	46%
	A	143	NH ₂	OCH ₃	OCH ₃	H	31%
	A	144	OH	OCH ₃	OCH ₃	OCH ₃	40%
	A	145	NH ₂	OCH ₃	OCH ₃	OCH ₃	30%
	A	146	OH	N(CH ₃) ₂	H	H	48%
	A	147	NH ₂	N(CH ₃) ₂	H	H	36%
	A	148	OH	CO ₂ Et	H	H	31%
	A	149	NH ₂	CO ₂ Et	H	H	66%
	B	150	OH				33%
	B	151	NH ₂				46%

The benzimidazole-4-carboxylates were prepared by reaction of 103 with the appropriate mono-, di- or tri-substituted benzaldehyde derivative. The yields given for the synthesis of the benzimidazole-4-carboxylates are with respect to compound 103, and the yields for the benzimidazole-4-carboxamides with respect to the corresponding carboxylates.

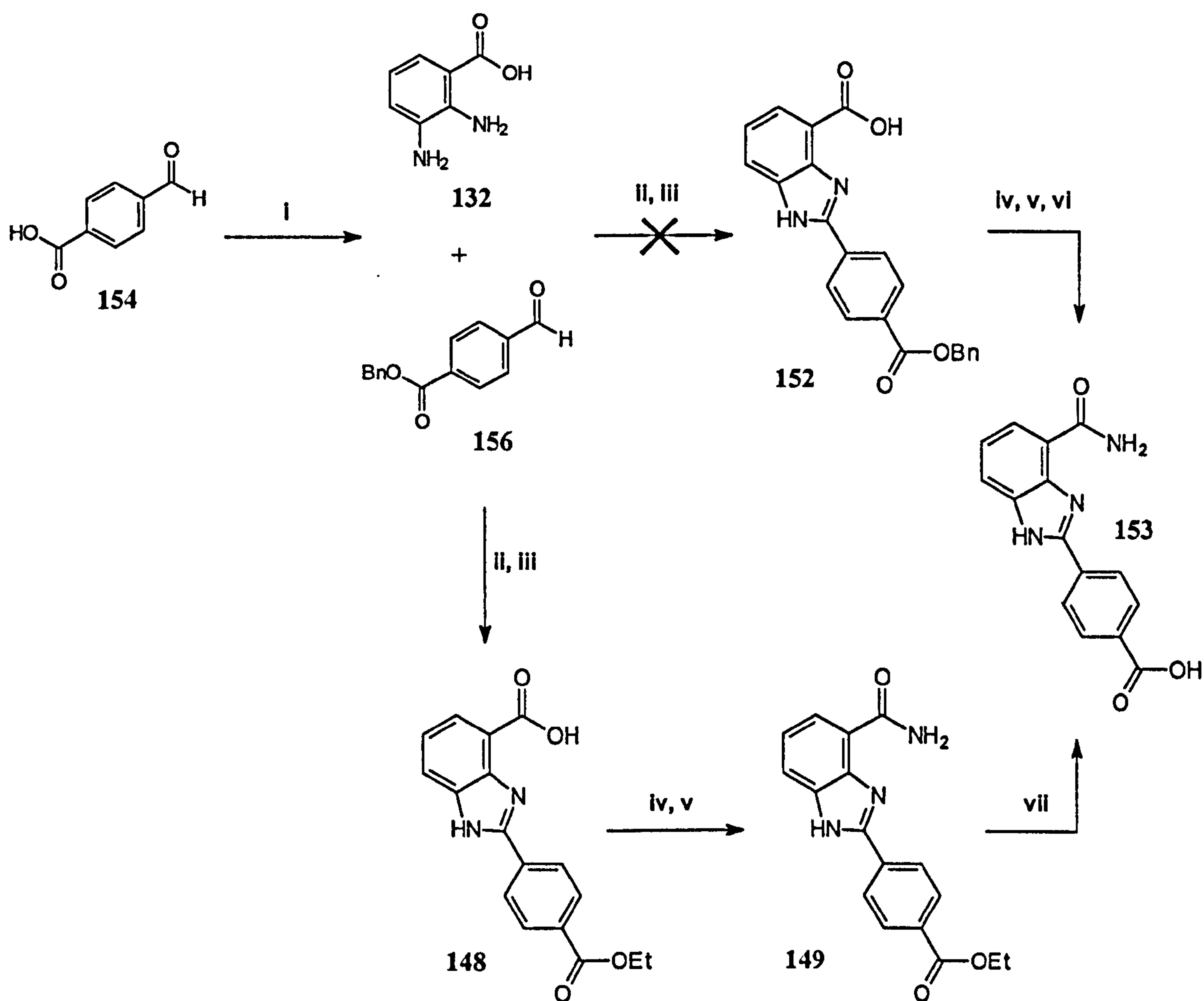
In most cases, the requisite benzimidazole-4-carboxylate readily precipitated from the crude aqueous reaction mixture. However, the presence of polar functionalities, such as hydroxyl groups, marginally increases the solubility of the compound in aqueous media and di- or trihydroxyl substituted benzimidazoles may be more appropriately synthesised directly from the corresponding di- or trimethoxy substituted benzimidazoles.

Compound 148 was obtained during the attempted synthesis of 2-(4'-carboxybenzylphenyl)-1*H*-benzimidazole-4-carboxylate 152. The 4'-carboxylate group, masked as a benzyl ester, would allow conversion of the 1-carboxylate to a carboxamide and subsequent deprotection would give 2-(4'-carboxyphenyl)-1*H*-benzimidazole-4-carboxamide (*Scheme 6.5.5.2*).

4-Carboxybenzaldehyde 154 was benzylated using *N,N*-diisopropyl-*O*-benzyl isourea 155, which was prepared from benzyl alcohol and *N,N*-diisopropyl carbodiimide.³⁰⁵ The reaction of 132 with 4-benzyloxycarbonyl benzaldehyde 156 led to the formation of 2-(4'-ethoxycarbonylphenyl)-1*H*-benzimidazole-4-carboxylate 148, possibly due to acid or copper-catalysed ester exchange. 148 was converted to the carboxamide 149 in 66% yield. In this case, the carboxylate is masked as an ethyl ester and still allows the selective conversion of the carboxylate in the 1-position. Unfortunately, time did not permit the deprotection of compound 149 to give the mono-acid 153.

The successful synthesis of the benzimidazole-4-carboxamides has expanded the existing series to include di- and tri-substituted derivatives. The copper-catalysed method of benzimidazole synthesis gives reasonable yields compared to the original proposed synthetic route and appears to be amenable to scale-up.

Scheme 6.5.5.2 *Attempted synthesis of 2-(4'-carboxyphenyl)-1H-benzimidazole-4-carboxamide 153*

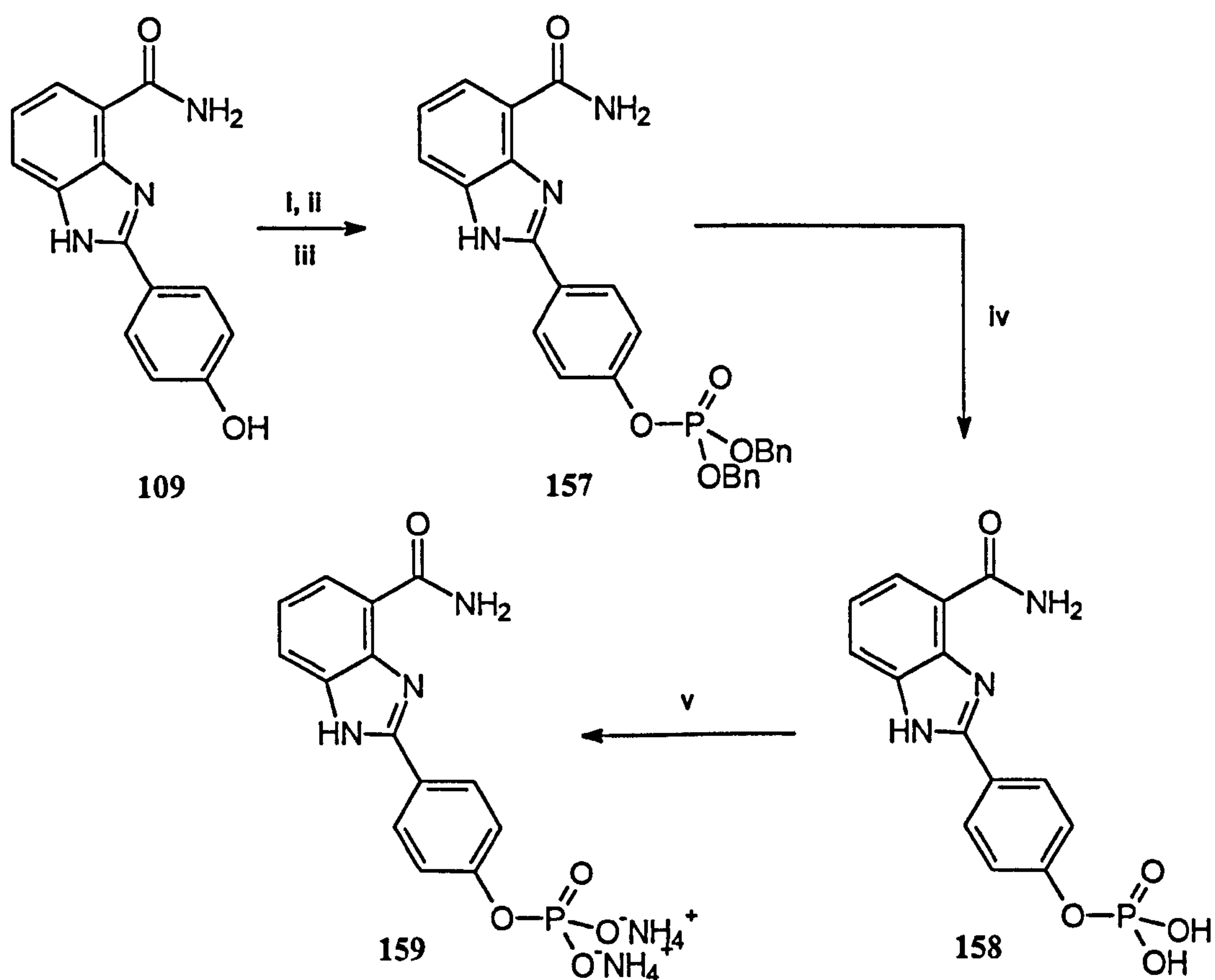


Reagents : i Copper (I) chloride, 0 °C, ii Cu(OAc)₂, MeOH, iii Na₂S, HCl, iv SOCl₂, DMF, dry THF, v aq. ammonia, vi H₂/Pd/C, MeOH, vii KOH, MeOH.

6.6 Synthesis of Benzimidazole Prodrugs

It was hoped that combining the improved method for the synthesis of **109** and the one-pot phosphorylation procedure developed for the quinazolinones,²⁸⁴ would lead to an increased yield of the benzimidazole phosphate prodrug. Phosphorylation of **109** was achieved in an analogous manner to the phosphorylation of the quinazolinone **37**, to give the dibenzyl phosphate ester **157** in 66% yield (*Scheme 6.6*).

Scheme 6.6 Synthesis of benzimidazole phosphate prodrugs



Reagents : i dry CCl_4 , dry MeCN -10°C , ii dry $\text{EtN}(\text{iPr})_2$, DMAP, -10°C , iii, dibenzyl phosphite, -10°C , iv THF:water (1:1), $\text{H}_2/\text{Pd/C}$, v aq. NH_4OH .

The yield previously recorded for the synthesis of **157** was only 29%.²⁵¹ Thus, the improved one-pot phosphorylation procedure led to a significant increase in the yield of the dibenzyl phosphate ester **157**. Deprotection of the dibenzyl ester was effected using a 1:1 mixture of redistilled THF and water, by hydrogenation in the presence of 10% Pd/C catalyst.²⁸¹ However, the required phosphate was found to be only sparingly soluble in aqueous media and was converted to the ammonium salt **159** in 74% yield. It is hoped that synthesis of the phosphate prodrugs will be amenable to scale-up as, unfortunately, time did not permit large-scale reactions to be carried out.

6.7 Summary

An improved method has been found for the synthesis of novel benzimidazole-4-carboxamides. The method gives reasonable yields and has allowed the synthesis of mono-, di- and tri-substituted derivatives. The one-pot phosphorylation procedure has been successfully applied to the synthesis of the benzimidazole prodrugs with increased yields. The benzimidazole prodrug was isolated as an ammonium salt to increase its aqueous solubility properties.

Chapter Seven: Biological Results and Discussion

7.1 Introduction

Biological studies were conducted on the quinazolinone series of compounds by members of the Cancer Research Unit, Medical School, University of Newcastle upon Tyne. PARP inhibitory activity was determined in permeabilised murine leukaemia L1210 cells (Section 7.2.1). Potentiation studies were also undertaken using 8-hydroxy-2-methylquinazolin-4-[3H]-one, NU1025, (37) which was evaluated for its ability to potentiate cytotoxicity in L1210 cells together with a range of mechanistically diverse anticancer agents. Preliminary HPLC results from the evaluation of the quinazolinone phosphate prodrug 96, will also be presented.

7.2 Inhibition Studies with PARP Inhibitors

7.2.1 Permeabilised Cell Assay

Permeabilised L1210 cells were used for PARP inhibition assays, following a variation of the method of Halldorsson *et al.*³⁰⁶ The L1210 cells were permeabilised by exposure to hypotonic buffer and cold shock. The cells were then incubated at 26 °C for five minutes in isotonic buffer, with [^{32}P]NAD⁺, the inhibitor under investigation and a synthetic, palindromic oligonucleotide (CGGAATTCCG). The oligonucleotide was included in order to stimulate PARP activity, as it forms a double-stranded hairpin loop with a blunt end.³⁰⁷ Following incubation, the incorporation of [^{32}P]NAD⁺ into acid precipitable material was estimated. Polymer precipitation was effected by the addition of 1% trichloroacetic acid (TCA). After collection of the precipitate and washing with 1% TCA and 1% sodium phosphate, the [^{32}P] incorporation into the precipitate was measured using a scintillation counter. The results are presented as the percentage of [^{32}P] uptake in drug-treated cells relative to the control cells. Inhibitory activity was expressed as an

IC₅₀ value (inhibitory coefficient value): defined as the concentration of inhibitor required to reduce PARP activity to 50% of the control.

7.2.2 96-Well Plate Assay

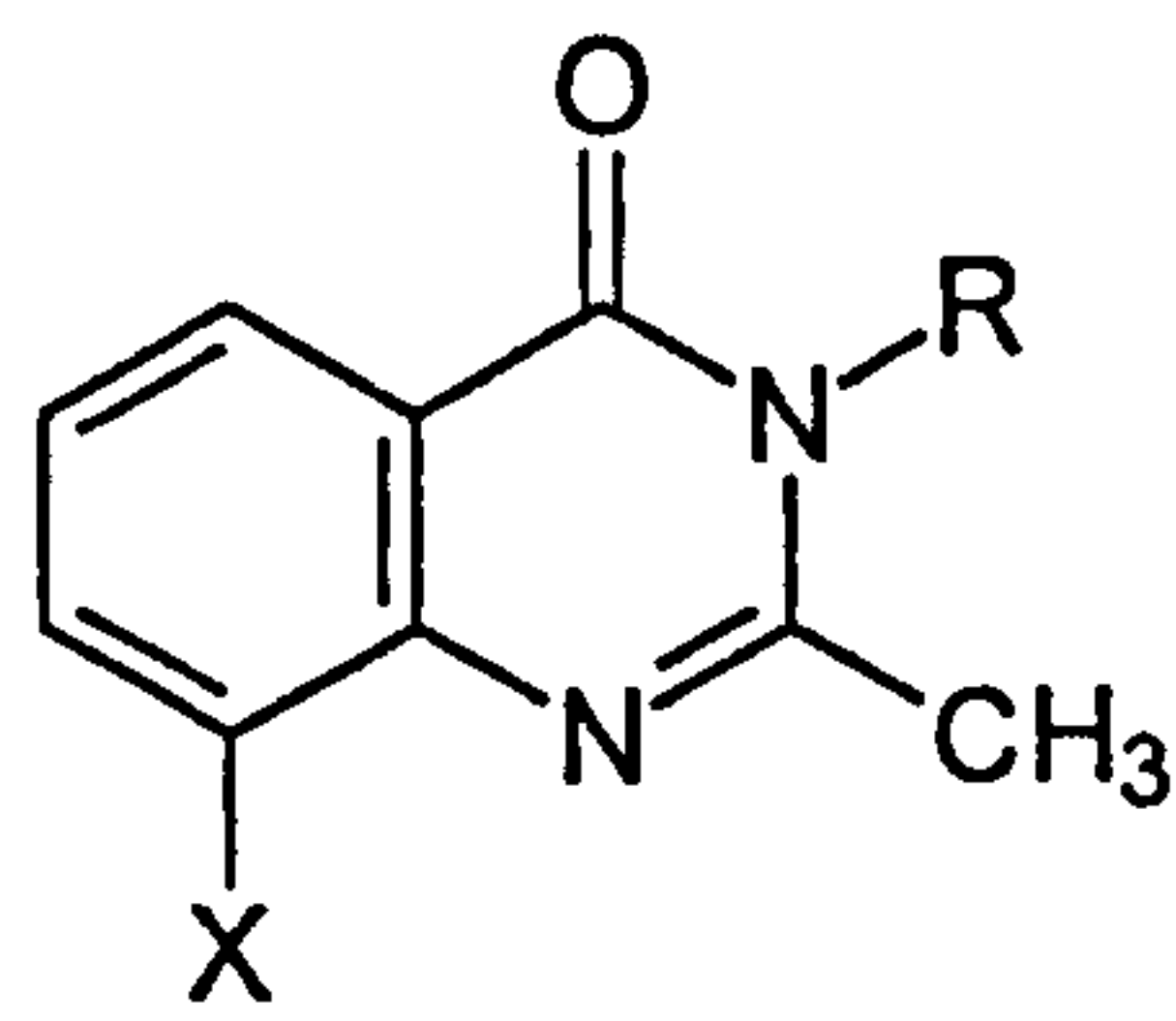
The benzimidazole inhibitors were evaluated by Agouron Pharmaceuticals Inc., San Diego, USA. Using a 96 well plate assay, the inhibitors were assayed at 25 °C, pH 8.0. The components of the assay included 50 mM Tris (Tris[hydroxymethyl]aminomethane), 10 mM MgCl₂, 1 mM Dithiothreitol (DTT), 8 µM/ml DNA, 500 µM [³²P]NAD⁺, 20 nM PARP and the inhibitor under investigation. The assays were run in triplicate and the reactions were terminated by the addition of 1% TCA solution. The mixtures were transferred to glass fiber filter well plates, vacuum filtered and washed. Activity was quantitated using a phosphorimager.

7.3 Results of Inhibition Studies with Quinazolinones

The inhibitory activities of 8-hydroxy-2-methylquinazolin-4-[3H]-one, NU1025, (37) and 8-hydroxy-2-(4'-nitrophenyl)quinazolin-4-[3H]-one, NU1057, (38) were previously determined using the permeabilised L1210 cell assay.^{263,264} IC₅₀ values of 0.44 ± 0.13 µM and 0.23 ± 0.033 µM were determined for compounds 37 and 38, respectively, indicating that the 4'-substituted aryl quinazolinone is slightly more potent than the 2-alkyl quinazolinone.³⁰⁸ Both compounds are an order of magnitude more potent than benzamide 6 and 3-hydroxybenzamide 10, which were found to have IC₅₀ values of 19.1 ± 5.9 µM and 13.7 ± 6.9 µM, respectively, under the same assay conditions.²⁶⁴

Figure 7.3.1 shows the results of inhibition studies on 2-alkylquinazolinones. The 2-alkyl quinazolinones 46, 48 and 37 show good activity against PARP. The interactions between these compounds and the enzyme may be attributed to a number of effects, including lipophilicity, electronic and steric effects.

Figure 7.3.1 Results of inhibition studies on 2-alkylquinazolinones

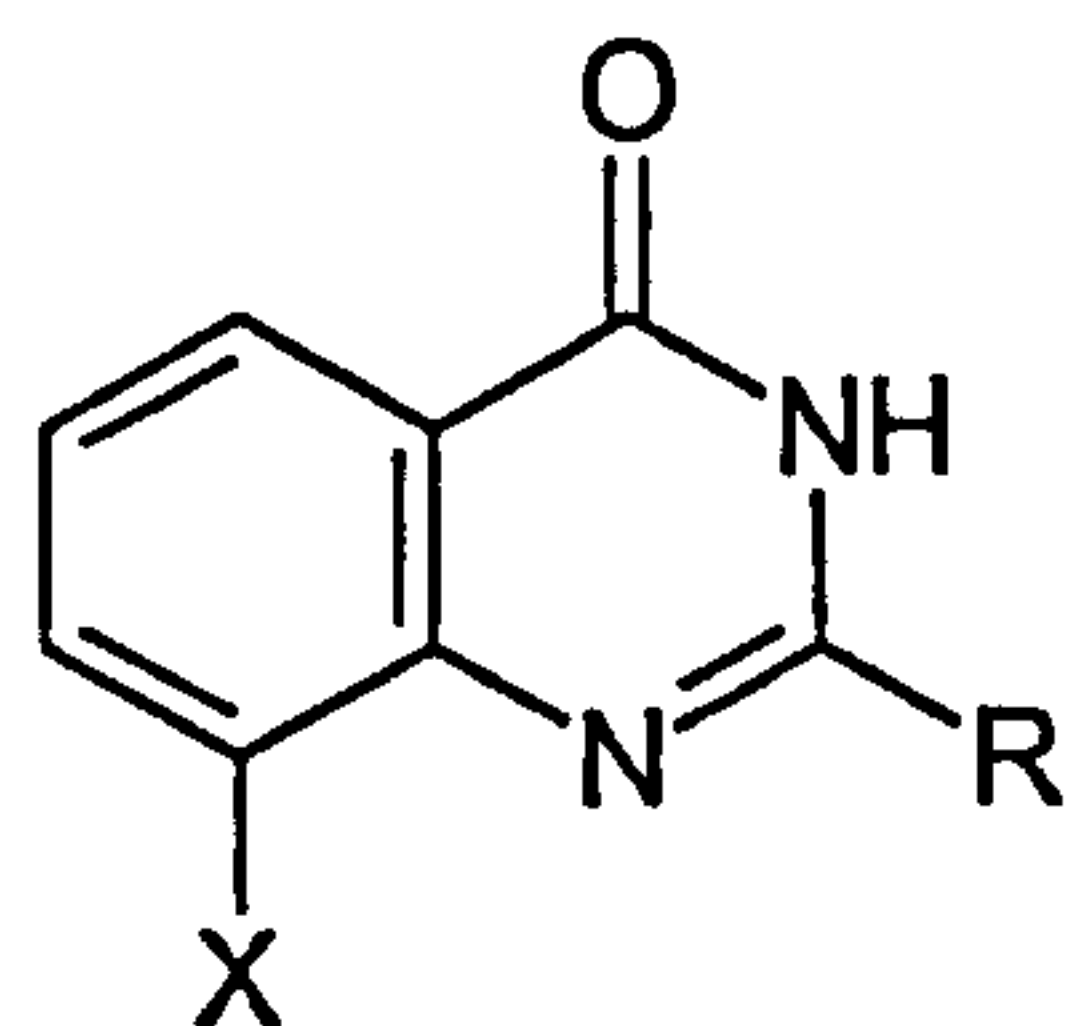


Compound No. (House No.)	Structure		IC ₅₀ value (μM)
	X	R	
48 (NU1069)	CH ₃	H	0.39
37 (NU1025)	OH	H	0.44
46 (NU1063)	OCH ₃	H	0.78
91 (NU1088)	OCH ₃	CH ₃	>100
92 (NU1055)	OH	CH ₃	8% inhibition at 10 μM

As expected, the two control compounds, 8-methoxy-3-*N*-methyl-2-methylquinazolin-4-[3*H*]-one 91 and 8-hydroxy-3-*N*-methyl-2-methylquinazolin-4-[3*H*]-one 92, were found to be weakly active as PARP inhibitors, with 91 having an IC₅₀ value of >100 μM, and 92 causing only 8% inhibition at 10 μM.

Figure 7.3.2 shows the results of inhibition studies on 2-arylquinazolinones.

Figure 7.3.2 Results of inhibition studies on 2-arylquinazolinones



Compound No. (House No.)	Structure		IC ₅₀ value (μM)
	X	R	
57 (NU1087)	CH ₃		0.13
85 (NU1075)	CH ₃		0.15
87 (NU1095)	CH ₃		0.22
38 (NU1057)	OH		0.23
67 (NU1097)	CH ₃		0.27
86 (NU1094)	OH		0.29
89 (NU1083)	CH ₃		0.44

Figure 7.3.2 continued

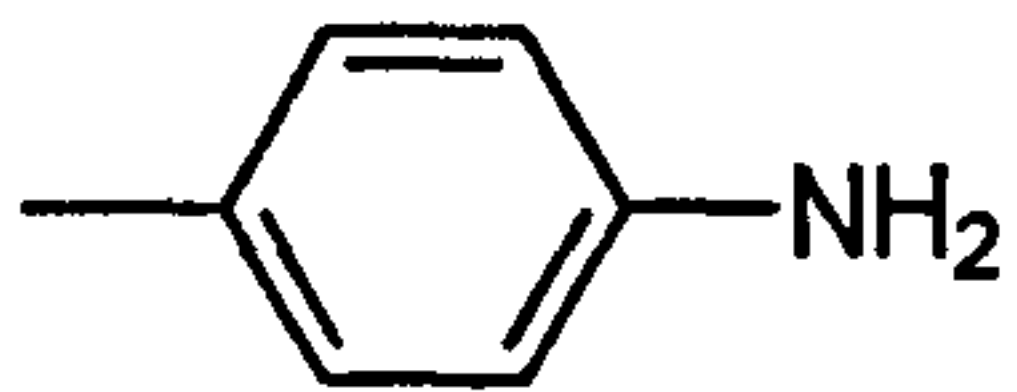
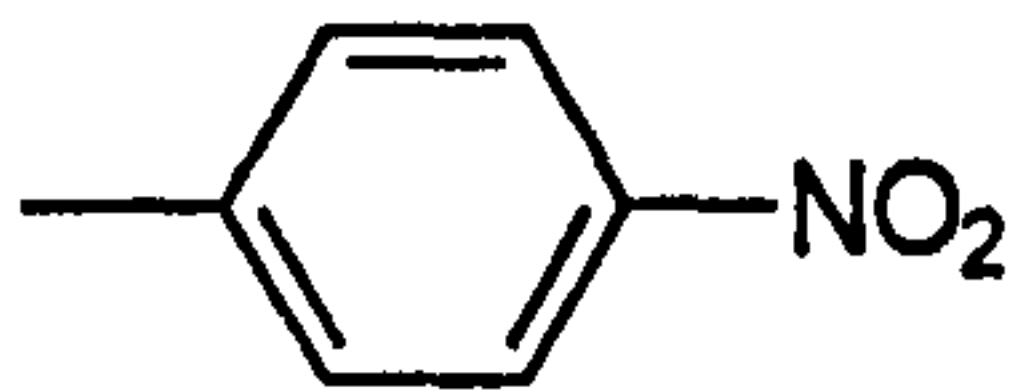
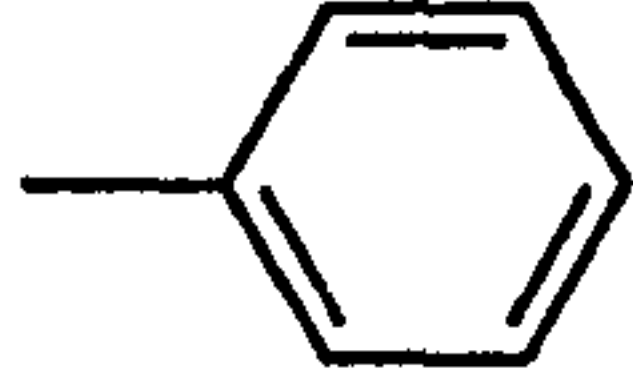
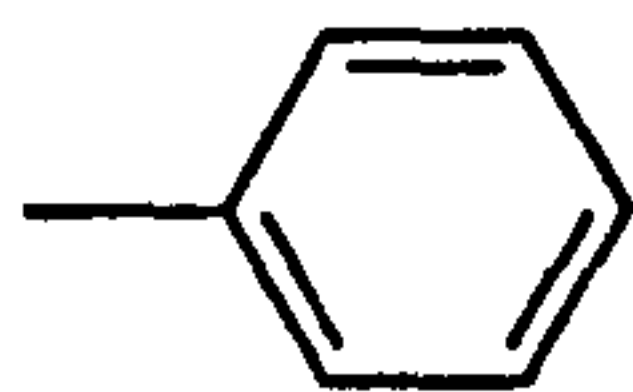
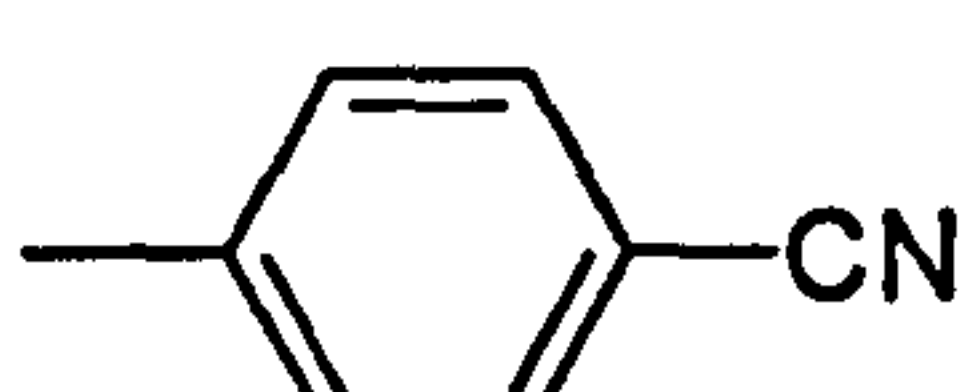
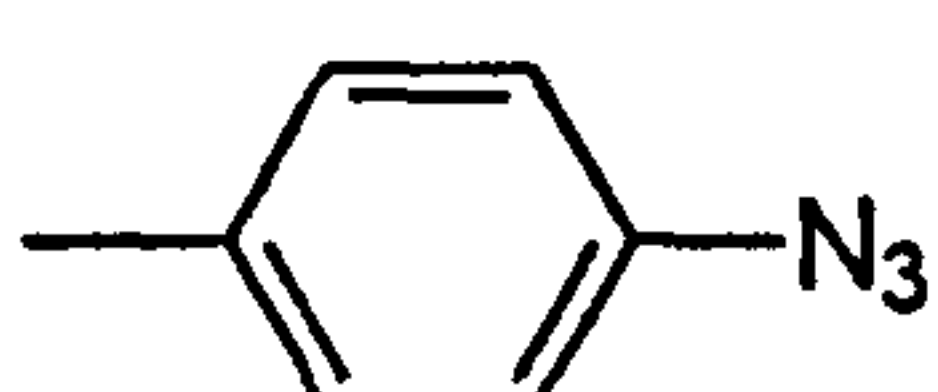
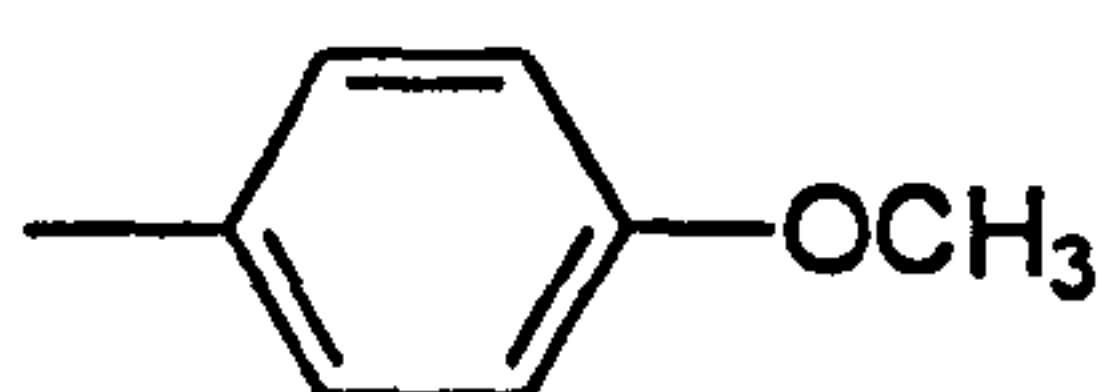
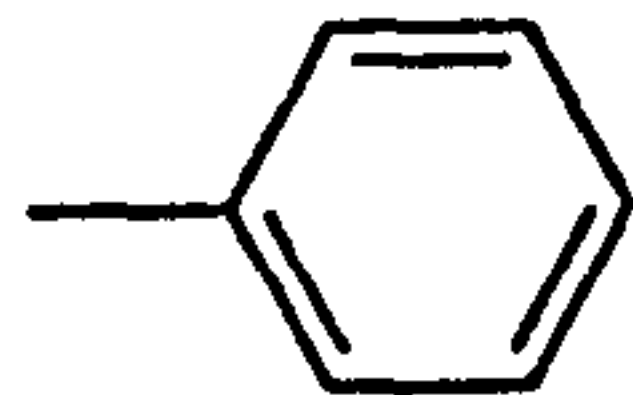
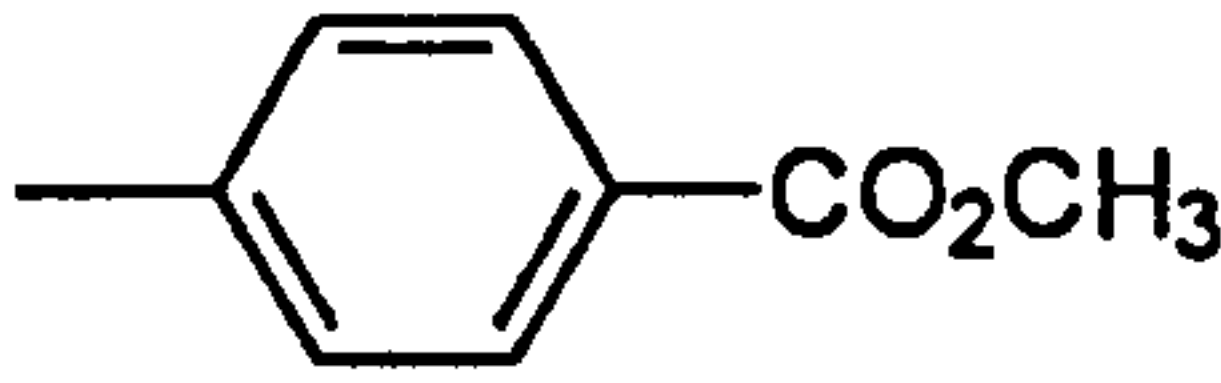
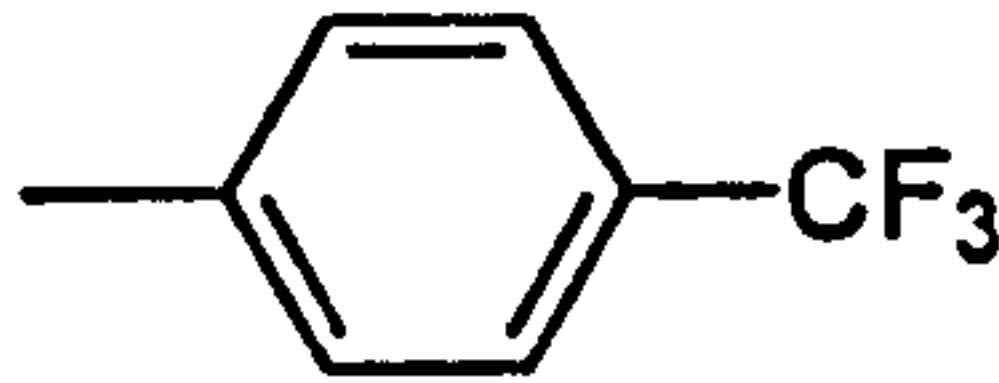
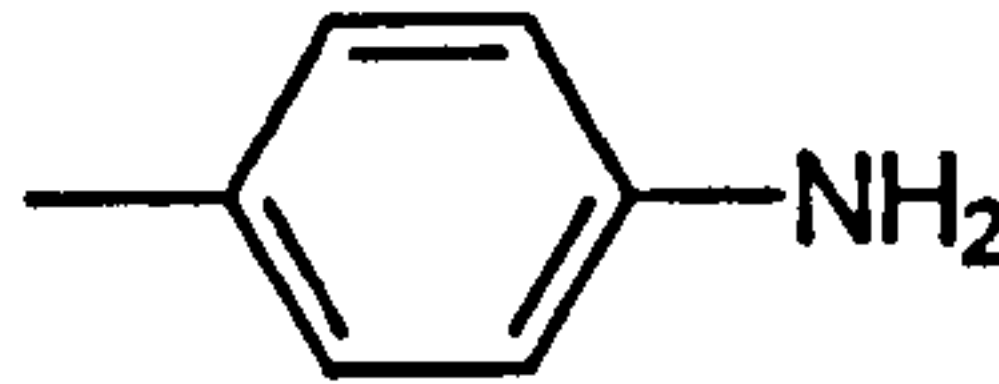
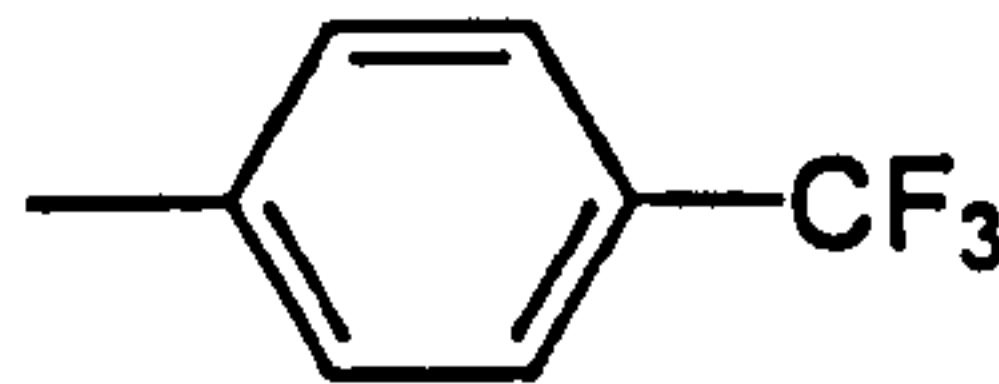
Compound No. (House No.)	Structure		IC ₅₀ value (μ M)
	X	R	
90 (NU1081)	OH		0.46
55 (NU1072)	OCH ₃		0.85
52 (NU1073)	CH ₃		0.87
53 (NU1068)	OH		1.06
65 (NU1089)	OCH ₃		1.34
80 (NU1106)	OCH ₃		1.93
84 (NU1071)	OCH ₃		2.0
51 (NU1065)	OCH ₃		4.2

Figure 7.3.2 continued

Compound No. (House No.)	Structure		IC ₅₀ value (μM)
	X	R	
76 (NU1108)	CH ₃		4.8
59 (NU1074)	OCH ₃		39
88 (NU1082)	OCH ₃		13% inhibition at 0.3 μM
61 (NU1078)	CH ₃		22.3% inhibition at 10 μM

The IC₅₀ values for the above compounds are in order of potency, starting with the most potent inhibitor. The introduction of a 2 phenyl substituent did not significantly increase the potency of the inhibitors compared to the 2-alkylquinazolinones. Again, the majority of the compounds are an order of magnitude more potent than benzamide 6. The insolubility of compounds bearing strongly electron-withdrawing substituents rendered the evaluation of some of these compounds difficult.

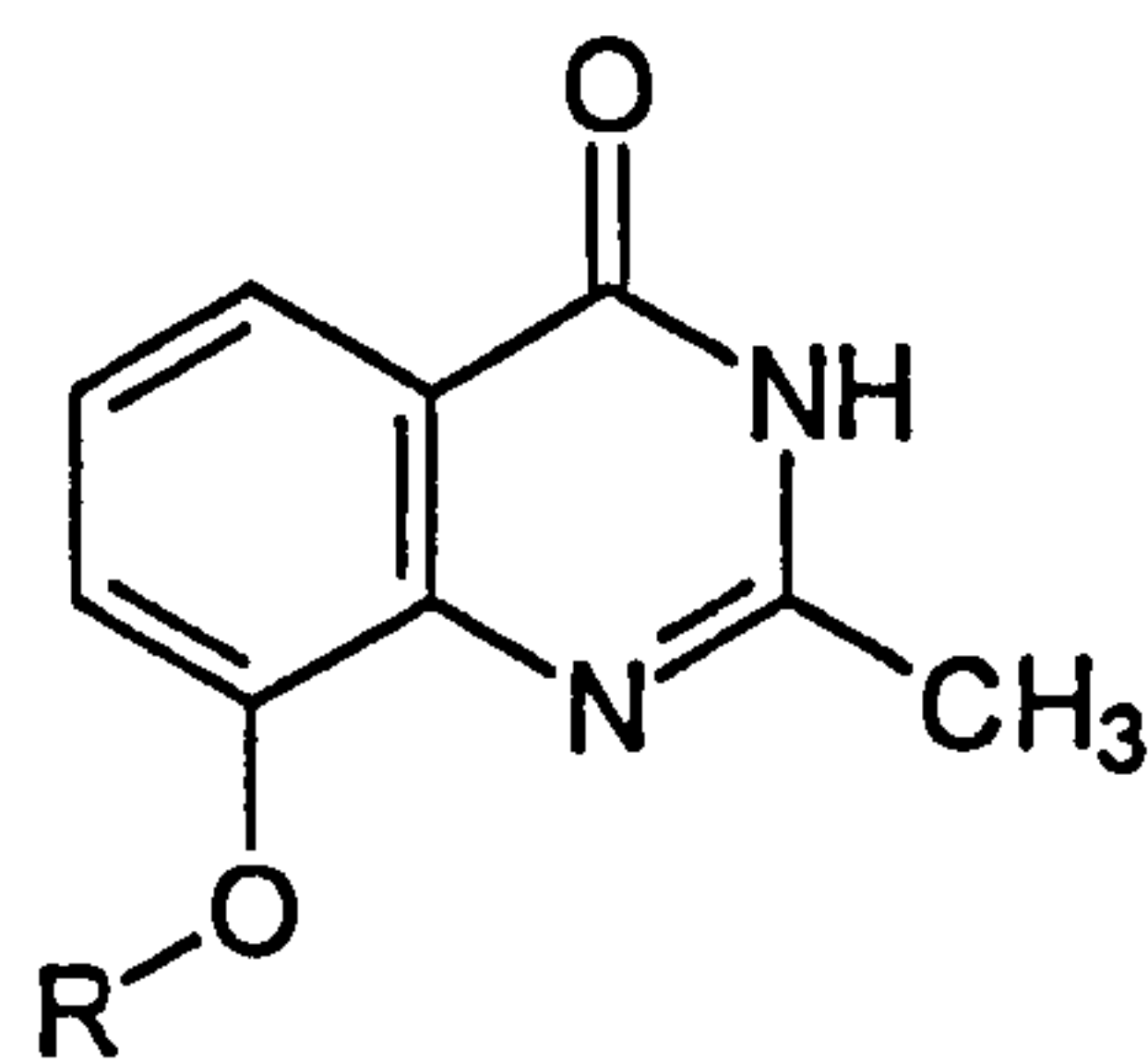
It is interesting to note that the derivatives 57 and 85 bear substituents of opposing electronic nature but are equipotent with respect to their PARP inhibitory activities, with IC₅₀ values of 0.13 and 0.15 μM, respectively. These results suggest that the electronic nature of the substituents on the aryl ring is not of primary importance for enzyme inhibitory activity.

Disappointingly, no clear structure-activity relationships have emerged from the evaluation of this series of compounds. However, previously determined structural requirements have been confirmed and this is reflected by the excellent inhibitory activity exhibited by the quinazolinones.

7.4 Evaluation of the Quinazolinone Prodrugs

The IC₅₀ values for the carbamate and phosphate quinazolinone prodrugs are shown below.

Figure 7.4 Results of inhibition studies on quinazolinone prodrugs



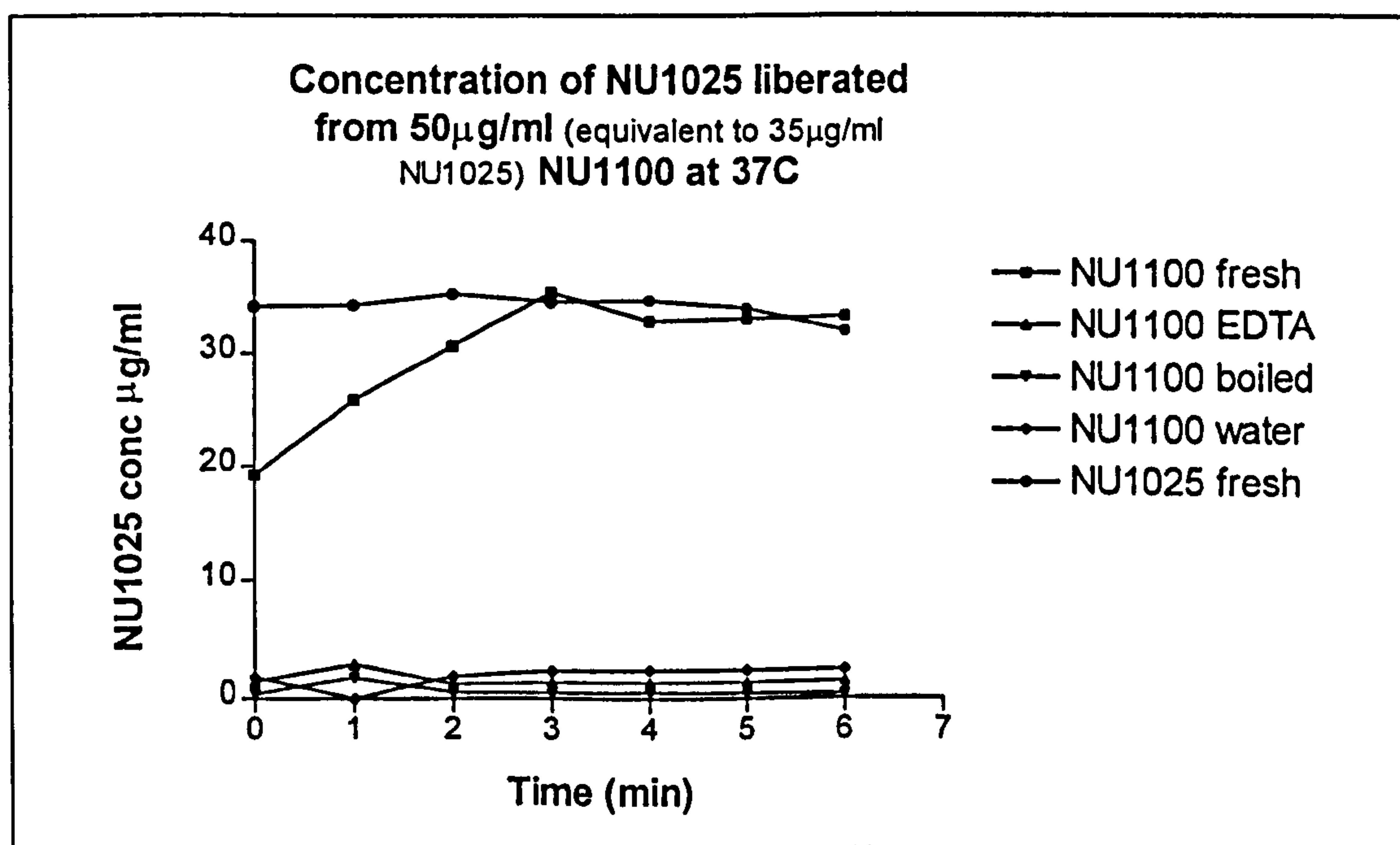
Compound No. (House Number)	Structure	IC ₅₀ value (μM)
	R	
93 (NU1084)		2.23
95 (NU1096)		Inactive at 1 μM
98 (NU1102)		> 100 μM

As previously described in Section 5.8, an ideal prodrug should be chemically stable and inactive. The glycine carbamate ester 93 was found to be moderately active against the PARP enzyme. However, the carbamate prodrugs were considered unsuitable derivatives, owing to their instability under physiological conditions. The more chemically stable phosphate esters 95 and 98 were found to be relatively inactive against the enzyme but the insolubility of these derivatives in aqueous media limits their utility.

7.4.1 Preliminary Results from *In Vivo* Studies

The quinazolinone prodrug NU1100 (96), was evaluated for its ability to liberate NU1025 (37) in plasma at 37 °C. Control studies showed that in pure water, pre-boiled plasma and EDTA-treated plasma, no liberation of NU1025 was evident, in agreement with expectations. In fresh plasma, the prodrug was converted to NU1025 in three minutes. *Figure 7.4.1.1* shows the data for the enzymatic breakdown of the quinazolinone prodrug 96 in plasma.

Figure 7.4.1.1 *Enzymatic breakdown of NU1100 (96) to NU1025 (37)*



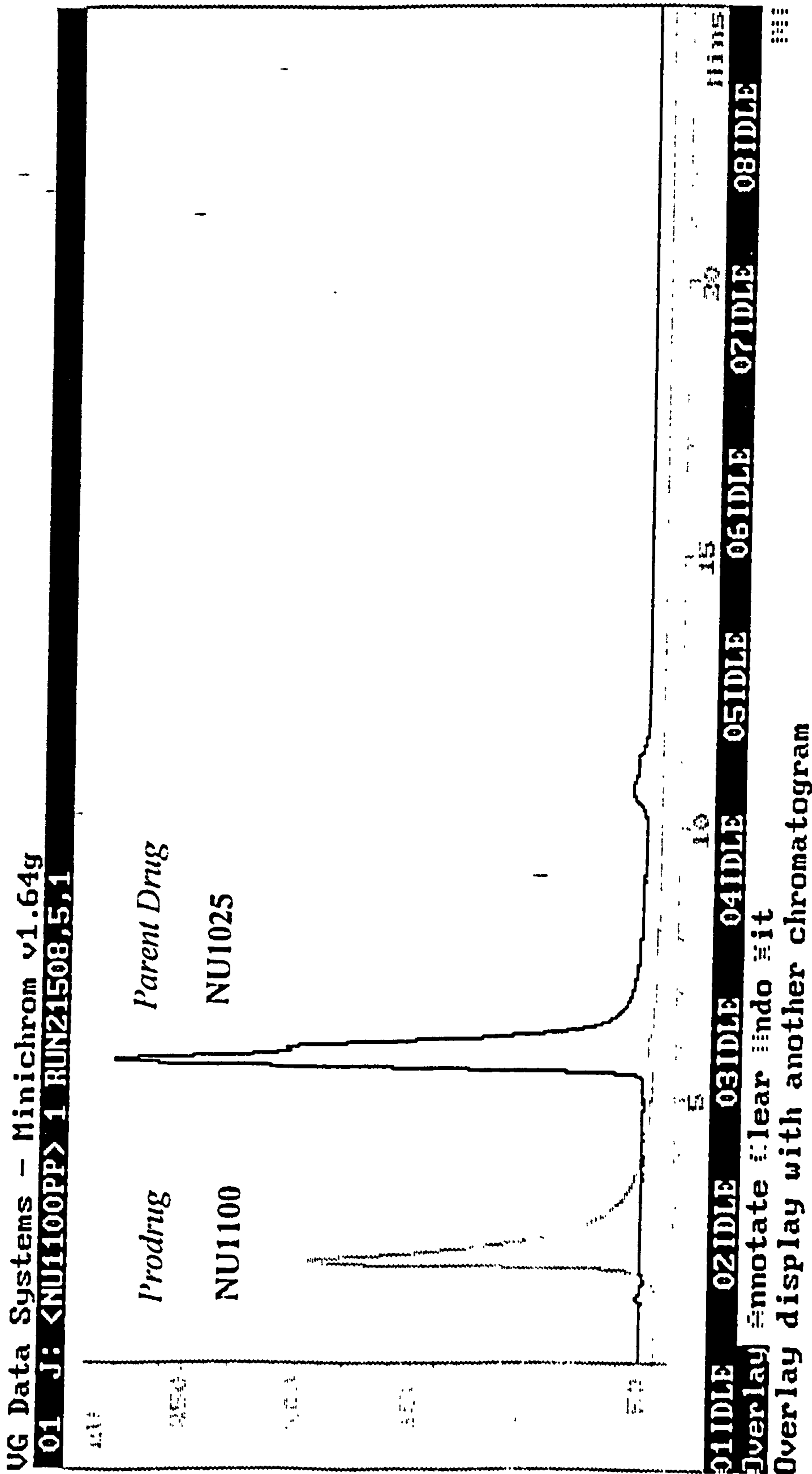
High Performance Liquid Chromatography (HPLC) Evaluation of the Quinazolinone Prodrug NU1100

Preliminary HPLC studies were conducted by Dr. J. A. Calvete, Cancer Research Unit, Medical School, University of Newcastle upon Tyne. Obvious problems were encountered with regard to the differing solubility properties of the prodrug NU1100 and the parent quinazolinone NU1025, as the stock solutions were prepared in aqueous and organic media, respectively.

Figure 7.4.1.2 shows an overlay of separate HPLC traces from the prodrug and parent drug, displaying different retention times. The run was carried out as a gradient assay, starting with 0% methanol in acetic acid and increasing to 20% methanol, over 10 minutes, with a flow rate of 1 ml/minute and sample injections of 3 µl.

Optimal HPLC conditions are currently being determined and validated. The aim of the study is to detect and quantitate the amount of prodrug and parent drug present under the *same* conditions and ultimately, to measure prodrug turnover *in vivo*.

Figure 7.4.1.2 HPLC traces from the prodrug NU1100 (96) and NU1025 (37)



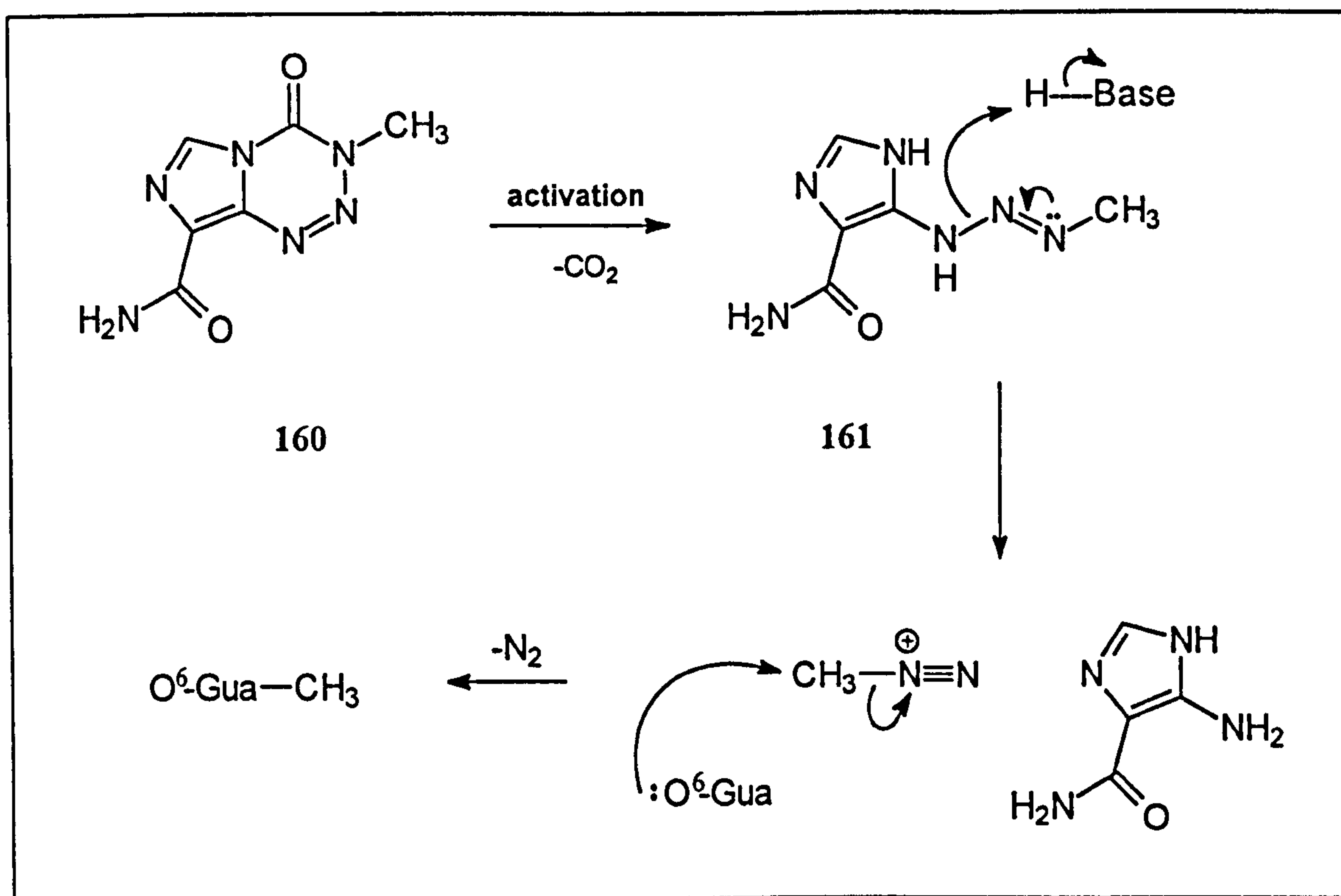
7.5 Results from Potentiation Studies

NU1025 (37) has been evaluated against a panel of diverse anticancer therapies, including γ -radiation, MTIC, bleomycin, AGG337, gemcitabine, camptothecin and etoposide (VP-16). The following sections will briefly overview the results from these potentiation studies.

7.5.1 Potentiation of Alkylating Agents

NU1025 (37) was evaluated in conjunction with the alkylating agents, temozolomide (8-carbamoyl-3-methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4-[3*H*]-one) 160 and MTIC 161. The chemically stable imidazotetrazinone 160 is presently undergoing clinical trials as an antitumour agent.³⁰⁹ The compound is activated *in vivo* by hydrolysis and loss of carbon dioxide, to yield the intermediate MTIC 161, believed to be the active form of temozolomide. *Figure 7.5.1* shows the mechanism of activation of temozolomide 160.³¹⁰

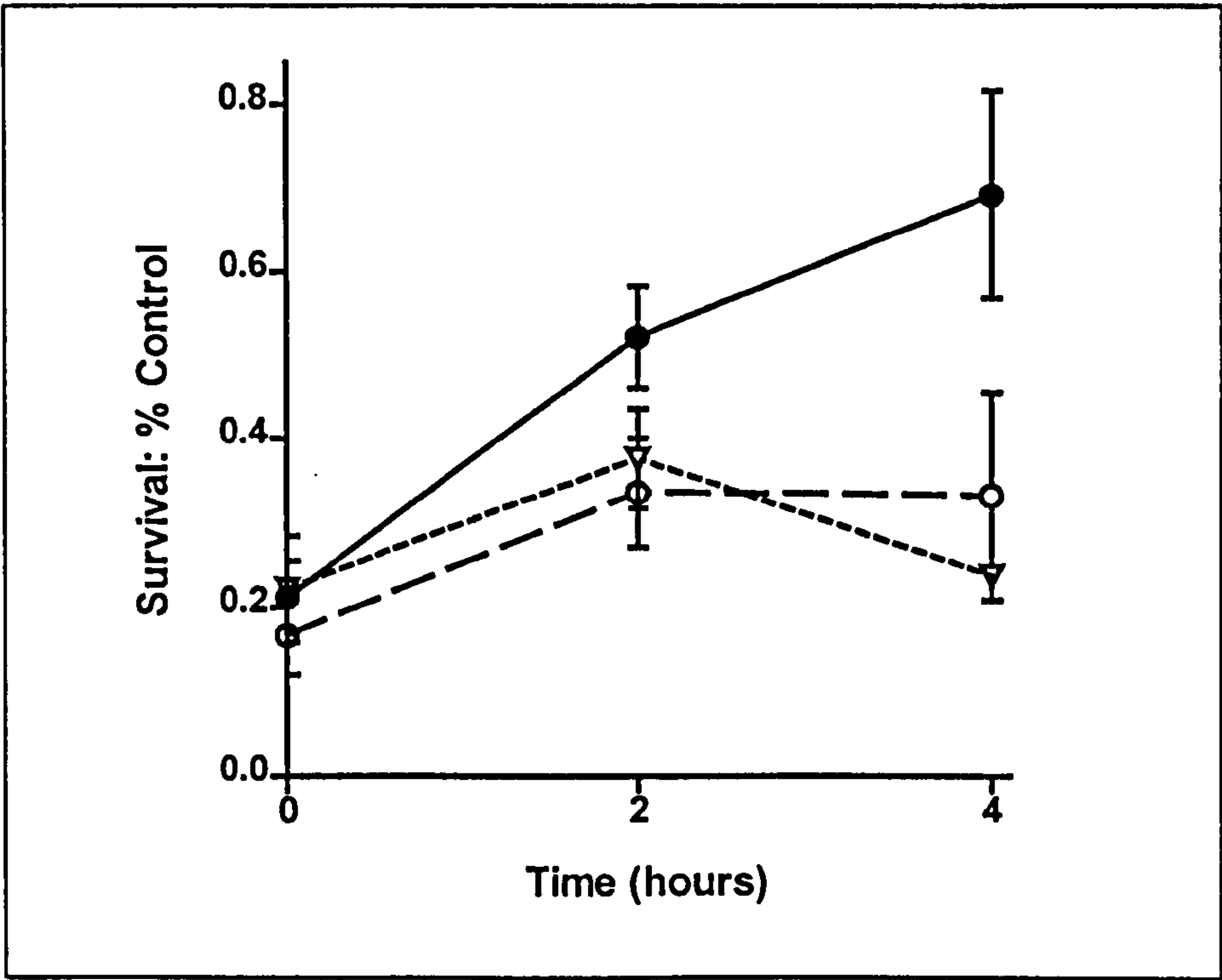
A study has been made using L1210 cells, comparing the effects of NU1025 (37) and PD128763 (30), with the less effective PARP inhibitors benzamide 6 and 3AB 8.²⁶⁴ The study showed a good correlation between the ability of the inhibitors to potentiate the effects of temozolomide and the potency of the compounds as PARP inhibitors. Micromolar concentrations of NU1025 and PD128763 were shown to give the same effects as millimolar concentrations of benzamide and 3AB, demonstrating a difference in concentrations of an order of magnitude. It was shown that 50-100 μ M NU1025 was required to potentiate the cytotoxic effects of MTIC (161) 4-7 fold, whereas 5 mM benzamide brought about only a 4-fold potentiation effect.²⁶⁴ These results suggest that NU1025 would be a possible candidate for clinical development, to be used in conjunction with anticancer agents and that MTIC also appears to be an appropriate cytotoxic agent for the pre-clinical evaluation of PARP inhibitors.

Figure 7.5.1 Proposed mechanism of activation of temozolomide 160³¹⁰

7.5.2 Potentiation of Ionising Radiation

The effects of NU1025 (37) and 3AB (8), on the repair of potentially lethal γ -irradiation-induced DNA damage were investigated. Exponentially growing L1210 cells were exposed to γ -irradiation, followed by a 2 hour recovery period, in the presence or absence of NU1025 or 3AB.³¹¹ As can be seen from *Figure 7.5.2.1* both NU1025 and 3AB potentiate the effects of γ -irradiation. The effects of 200 μ M NU1025 were more pronounced than 10 mM 3AB. It was also found that 10 mM 3AB alone caused a reduction in cell survival of $18 \pm 13\%$, compared to a $3 \pm 5\%$ reduction for NU1025, which may be due to cytotoxicity unrelated to the inhibition of PARP. *Figure 7.5.2.2* shows the effects of NU1025 and 3AB on the repair of potentially lethal damage (PLD).³¹¹

Figure 7.5.2.2 *The effects of NU1025 and 3AB on the repair of potentially lethal damage (PLD)*



Recovery from potentially lethal γ -irradiation damage in the presence/absence of NU1025/3AB. After exposure to 8 Gy γ -irradiation, cells were allowed to recover for up to 4 hours in control medium (●) or medium containing 200 μ M NU1025 (○), or 10mM 3AB (▽) prior to seeding for colony formation. Data are mean \pm SD of triplicate colony counts from each of the cell populations exposed in duplicate, from a single representative experiment.³¹²

Following exposure to 8 Gy γ -irradiation, the L1210 cells were allowed to recover in the presence/absence of NU1025/3AB. It was found that in the absence of the inhibitors slight recovery was evident, which was inhibited in the presence of 200 μ M NU1025 or 10 mM 3AB.³¹¹ Repair of γ -irradiation DNA damage was measured by alkaline elution. The results indicated that NU1025 hindered DNA repair. The additional cell killing observed with 3AB may, again be due to cytotoxicity unrelated to the inhibition of PARP.

7.5.3 Potentiation of Other DNA-Damaging Agents

7.5.3.1 PARP Inhibitors and Bleomycin

Bleomycin is an antitumour antibiotic which has been found to be particularly useful for the treatment of lymphomas, testicular carcinomas and squamous cell carcinomas.³¹² The drug inhibits prokaryotic and eukaryotic cell proliferation and DNA synthesis. Bleomycin-induced DNA fragmentation is predominantly single-stranded but double-strand breakages also occur.³¹² It has been hypothesised that one mechanism by which human tumours may become resistant to bleomycin is through increased DNA repair, although no exact mechanisms have been defined.³¹²

7.5.3.2 PARP Inhibitors and Antimetabolites

NU1025 failed to potentiate the cytotoxicities of the classical antifolate thymidylate synthase (TS) inhibitor, AGG337 and the antimetabolite, gemcitabine.³¹¹ The cytotoxicity of TS inhibitors is believed to involve BER, resulting from increased deoxy-uridine-triphosphate (dUTP) pools and the mis-incorporation of uridine into DNA. The subsequent removal of the wrongly incorporated base by a specific BER enzyme leads to a DNA strand breakage and ultimately cell death.³¹² Failure of NU1025 to potentiate the effects of antimetabolites suggests that these drugs may not be suitable for use in conjunction with PARP inhibitors.

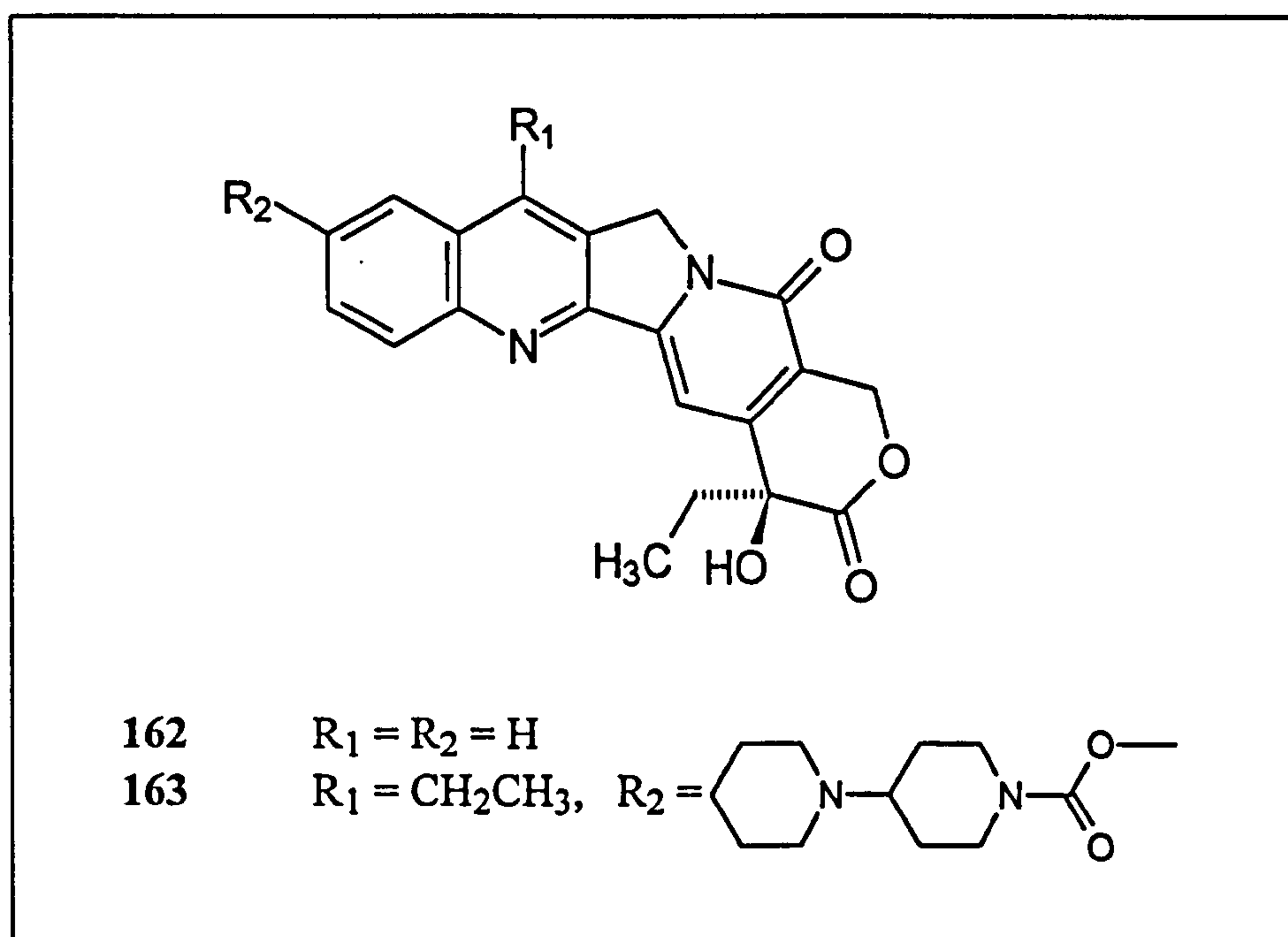
7.5.3.3 PARP Inhibitors and Topoisomerase Inhibitors

The topoisomerase enzymes (Class I and II) are responsible for the catalysis of a three-step process:³¹³

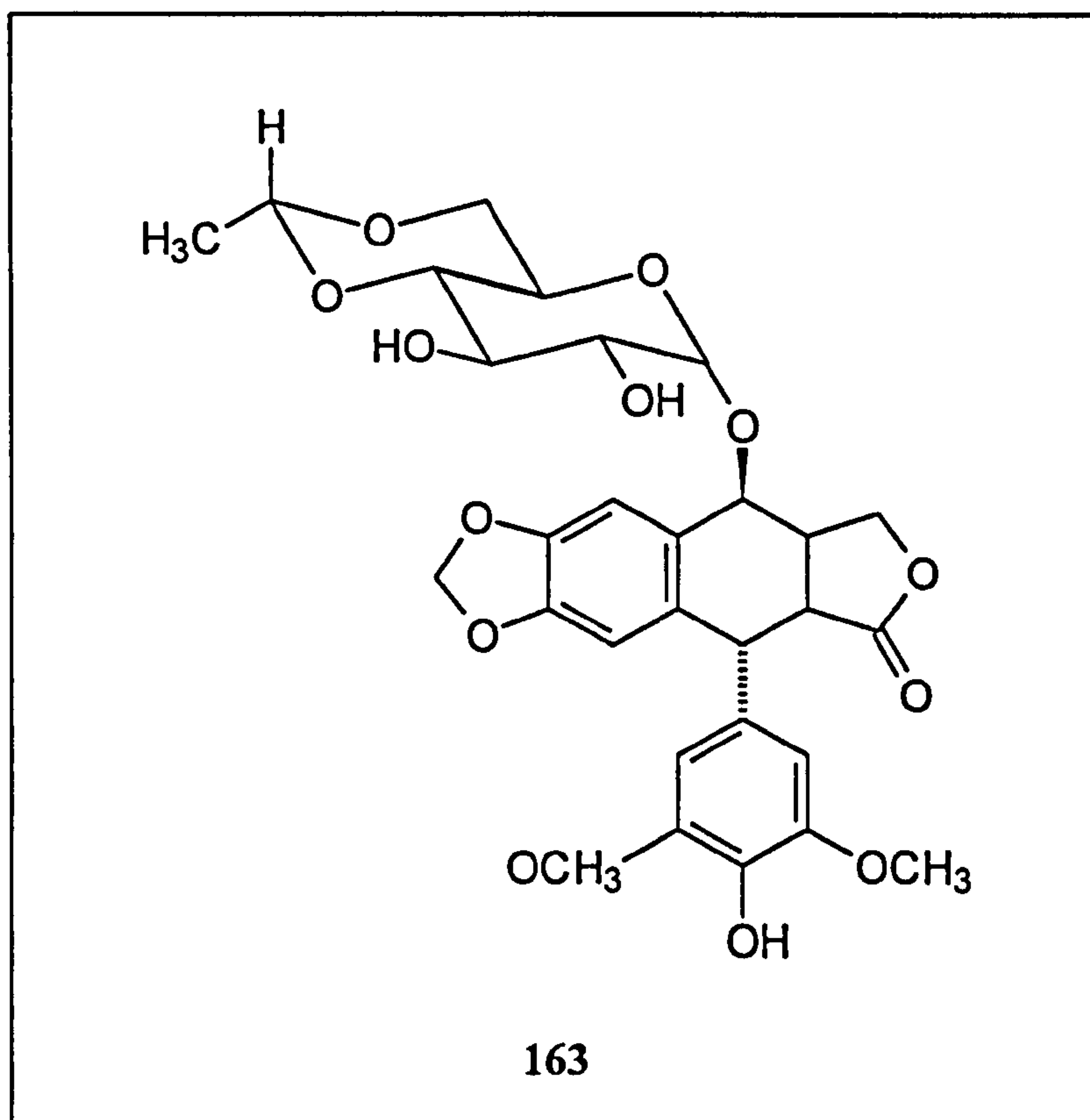
- i) cleavage of one (topo I) or both (topo II) strands of DNA,
- ii) the passage of a segment of DNA through the break, and
- iii) resealing of the break.

Topoisomerase I Inhibitors

The only currently known drugs in use as topoisomerase I (topo I) inhibitors are camptothecin and its derivatives.³¹² One of the more soluble analogues is CPT-11 (163), which is currently undergoing clinical trials.³¹² NU1025 was found to potentiate the cytotoxic effects of camptothecin.

**Topoisomerase II Inhibitors**

Etoposide (VP-16) 163 is an inhibitor of topoisomerase II (topo II) in eukaryotic cells, effective in the treatment of lung and testicular cancers.³¹² Topo II makes a double strand incision in DNA and forms a 'cleavable' complex (the complex is termed 'cleavable' since a permanent DNA double-strand break would result from the removal of the protein).³¹² Topo II inhibitors stabilise the cleavable complex and prevent any further enzymatic topological changes from occurring in the DNA. Resistance to etoposide commonly arises as the classical MDR-phenotype, characterised by overexpression of the levels of P-glycoprotein (see Section 1.2.2). NU1025 failed to potentiate the cytotoxic effects of etoposide (VP-16).



These results are in agreement with the findings of Ménissier de Murcia *et al*⁸⁸, but in contrast to the earlier work of Benjamin and Gill.⁷⁴ Since the inhibition of topo I or topo II would result in a permanent single or double-strand DNA break, respectively, the inhibition of PARP would be expected to potentiate the effects of topo I but not topo II inhibitors. *Table 7.5* summarises the results of the potentiation studies using NU1025 and a variety of DNA-damaging agents.

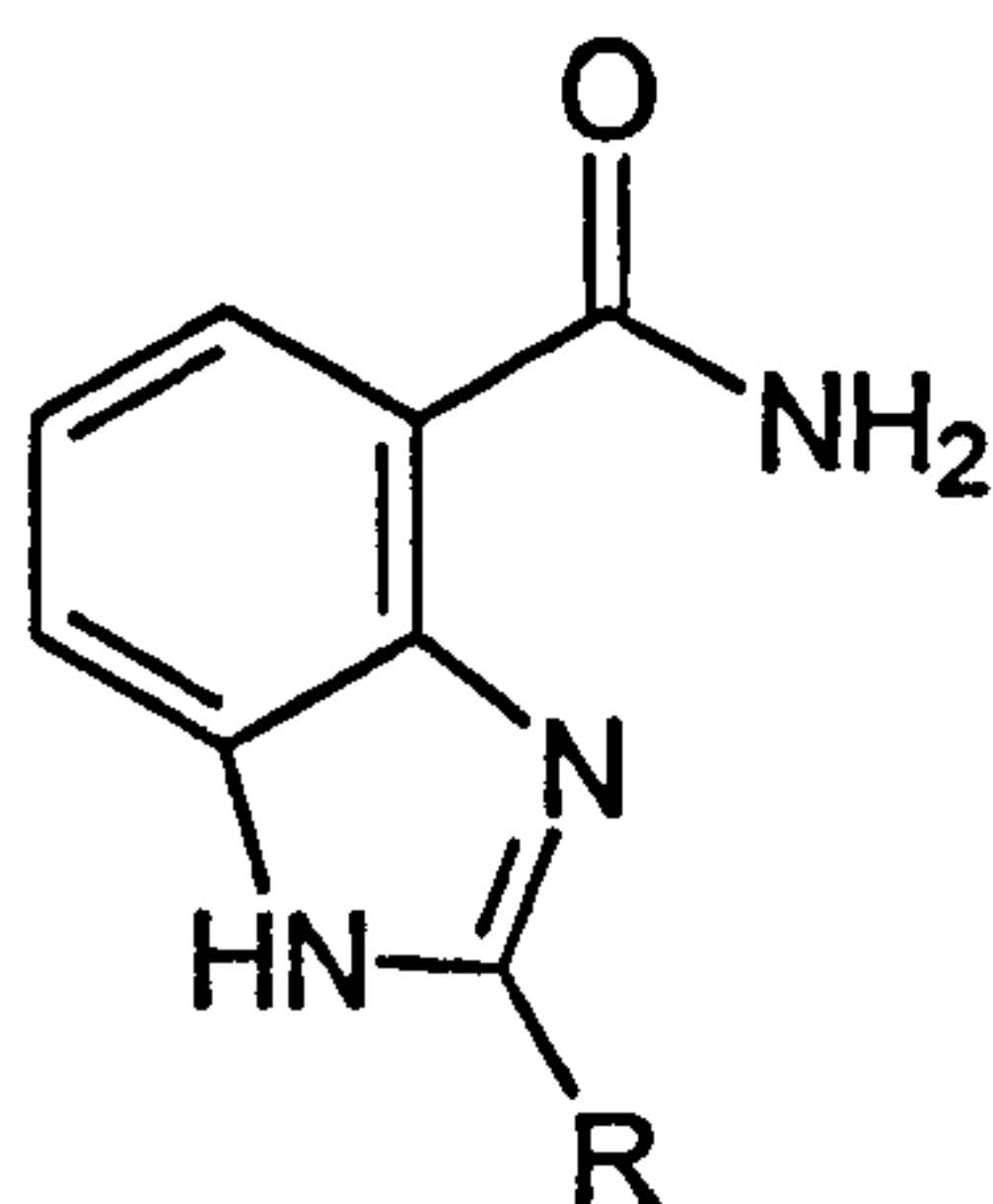
Table 7.5 *Results of potentiation studies using NU1025 (37)*

DNA-Damaging Agent	Fold Potentiation
MTIC	3.6 ± 0.5
Camptothecin	2.6 ± 0.3
Bleomycin	2.0 ± 0.5
γ -Irradiation	1.4 ± 0.2
AGG337	None
Gemcitabine	None
Etoposide	None

7.6 Results of Inhibition Studies with Benzimidazoles

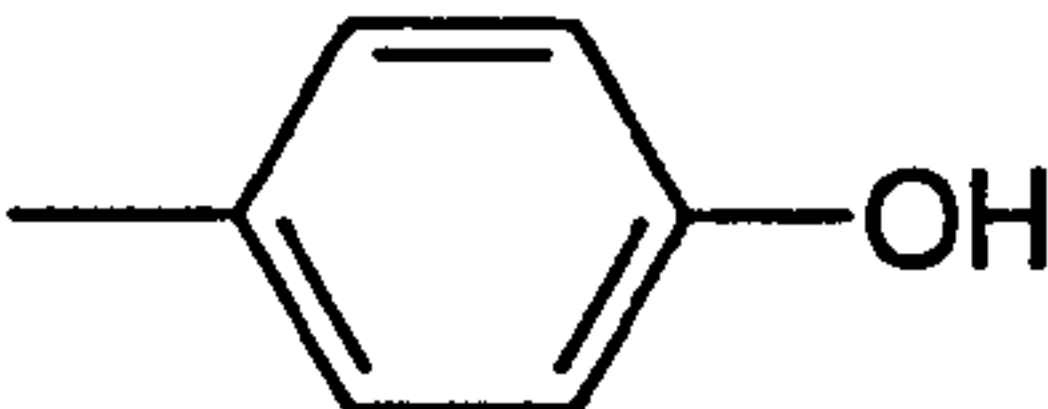
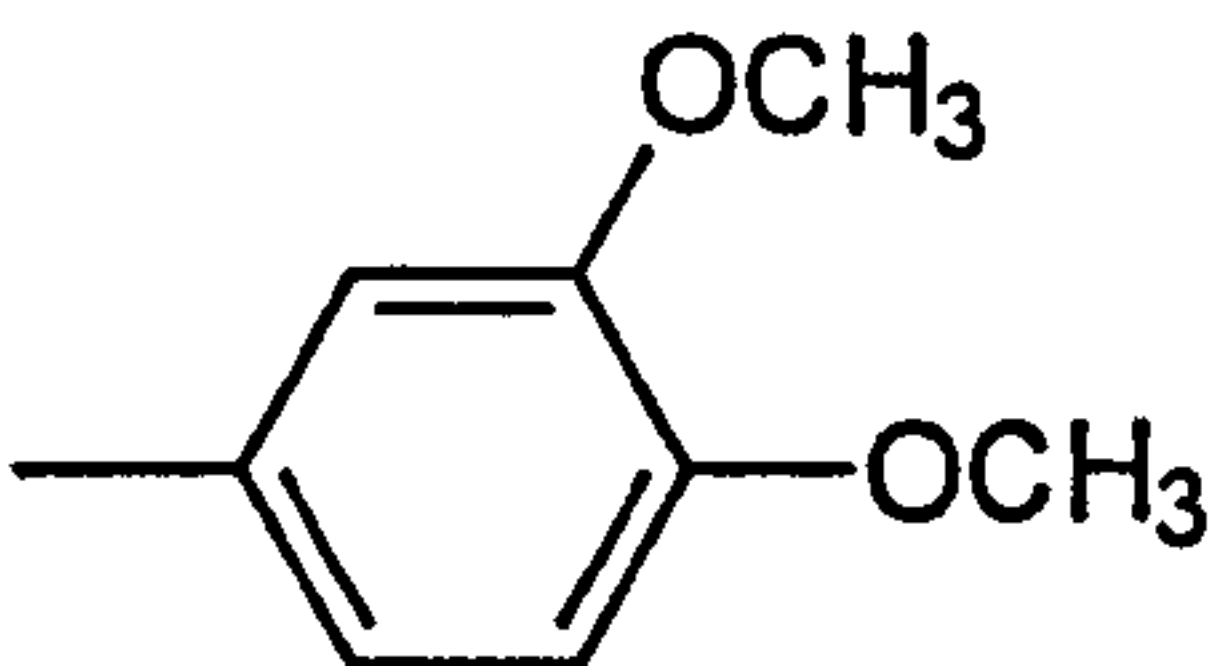
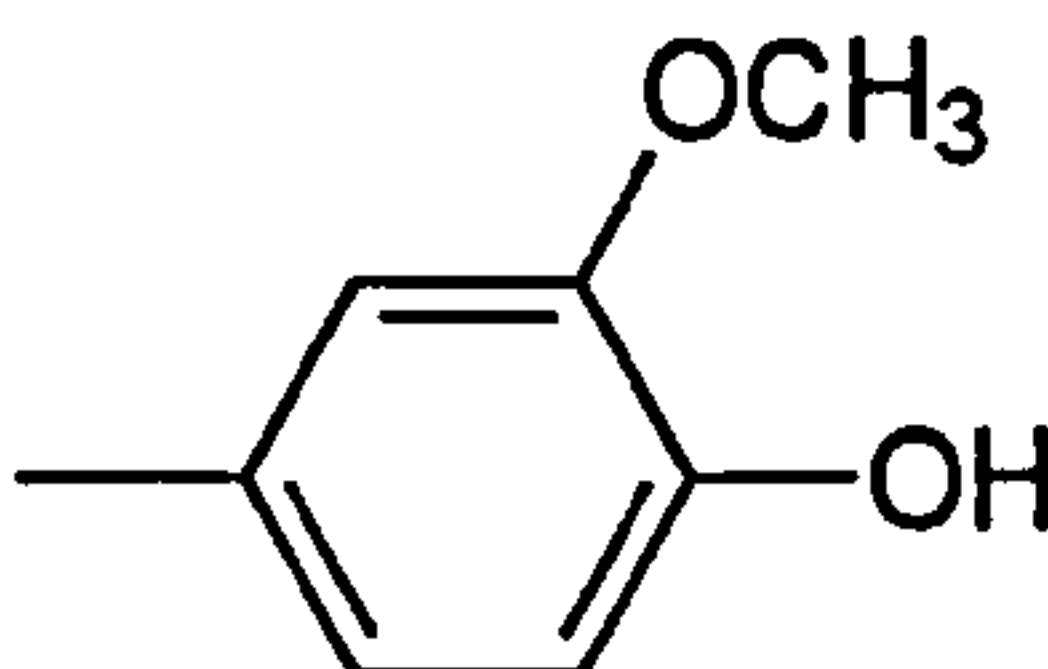
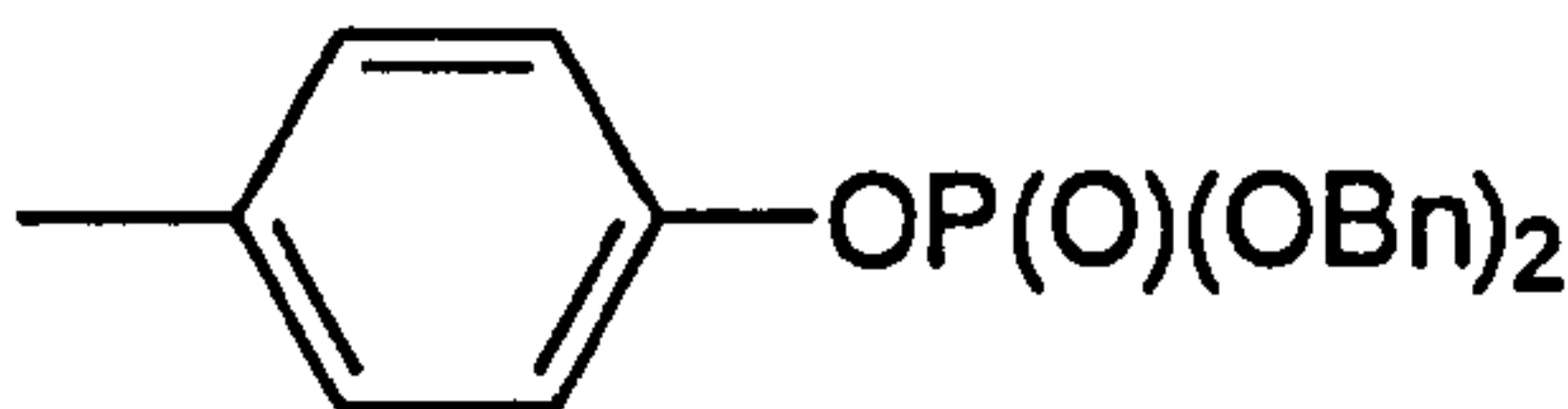
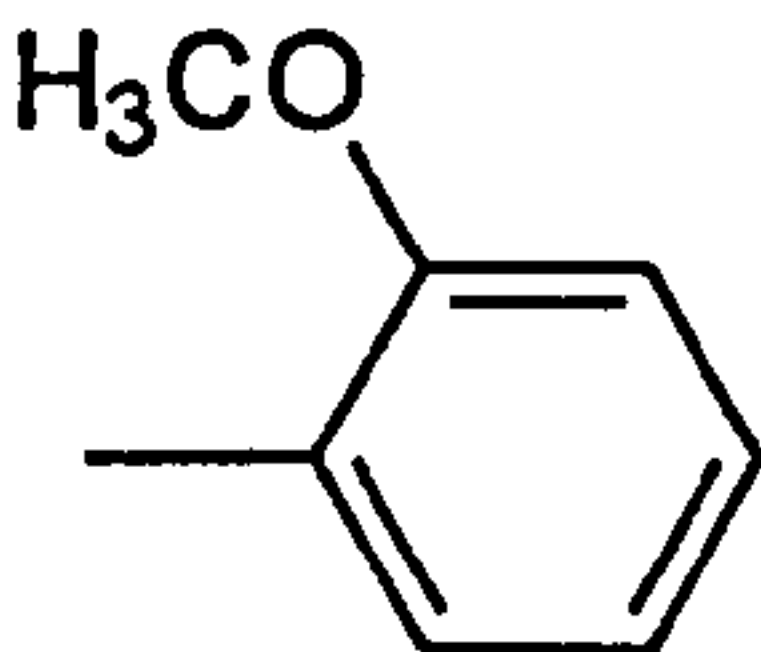
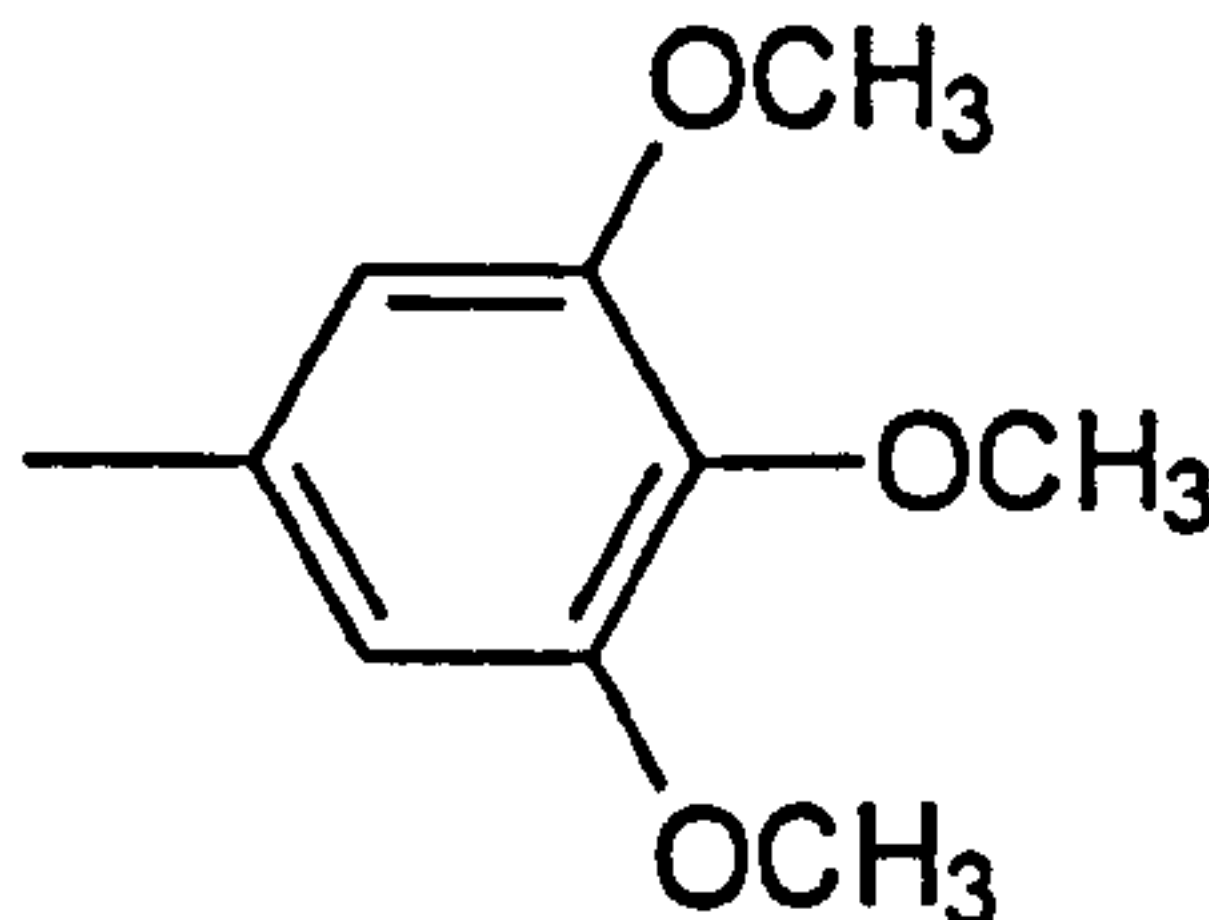
Figure 7.6.1 shows the results of inhibition studies using the benzimidazole-4-carboxamides. The K_i values given below are in order of potency, starting with the most potent. Unfortunately, no clear structure-activity relationships have emerged from the evaluation of the benzimidazole inhibitors. It appears that substitution in the *ortho* position of the 2-aryl ring is not well tolerated by the enzyme, as is reflected by the lower activity of NU1098 (137). Interestingly, the addition of a third methoxy group, as in compound 145 resulted in a substantial loss of activity, since the K_i values for the di- and tri-methoxyphenyl benzimidazoles, 143 and 145, are 6.2 nM and 128 nM, respectively. The benzimidazole derivatives clearly exhibit exceptional inhibitory activity against PARP, and are among the most potent inhibitors known to date.

Figure 7.6.1 Results of inhibition studies with benzimidazoles



Compound No. (House No.)	Structure	K_i value (nM)
	R	
134 (NU1109)	<p>Chemical structure of R for compound 134: A benzene ring with a chlorine atom (Cl) at the para position relative to the attachment point.</p>	3.3 ± 0.7
139 (NU1117)	<p>Chemical structure of R for compound 139: A benzene ring with a hydroxyl group (OH) at the para position relative to the attachment point.</p>	4.8 ± 0.8
147 (NU1111)	<p>Chemical structure of R for compound 147: A benzene ring with a dimethylamino group ($\text{N(CH}_3)_2$) at the para position relative to the attachment point.</p>	5.1 ± 0.7
149 (NU1116)	<p>Chemical structure of R for compound 149: A benzene ring with an ethoxycarbonyl group (CO_2Et) at the para position relative to the attachment point.</p>	5.3 ± 0.6
151 (NU1118)	<p>Chemical structure of R for compound 151: A benzene ring fused to a five-membered ring containing two oxygen atoms (a 2,3-dihydrobenzofuran derivative) at the 2-position.</p>	5.6 ± 0.4

Figure 7.6.1 continued

Compound No. (House No.)	Structure	K _i value (nM)
	R	
109 (NU1085)		6 ± 1.3
143 (NU1114)		6.2 ± 0.3
141 (NU1113)		13 ± 1.5
157 (NU1115)		66 ± 18
137 (NU1110)		124 ± 49
145 (NU1112)		128 ± 22

7.7 Results from Kinetic Experiments

Kinetic studies were conducted with the quinazolinone NU1025 (37) to determine the nature of PARP inhibition by the compound. The results showed that NU1025 increased the K_M for NAD^+ and decreased the V_{max} of PARP, which indicates that the predominant nature of the inhibitor is competitive but with a ‘mixed-type’ inhibition component.³¹¹ This was found to be the case using both permeabilised cell assays and purified ‘Super’ PARP* assays.³¹¹ *Figure 7.7* shows the K_M and V_{max} values determined for the NU1025 in the permeabilised cell and ‘Super’ PARP assays.³¹¹

Figure 7.7 Results of kinetic analysis of NU1025 (37)

i) Permeabilised cell assay:

	K_M (μM)	V_{max}
Control	617	1049
0.4 μM NU1025	854	760

ii) Purified PARP assay:

	K_M (μM)	V_{max}
Control	170	1115
0.3 μM NU1025	220	786

* Carries a mutation at residue Leu 713 (Leucine→Phenylalanine)

7.8 Summary

From the results of inhibition studies carried out with the quinazolinone derivatives, it is clear that the majority of the compounds are effective PARP inhibitors. The potency of compounds **57** and **85** indicate that the electronic nature of the 4'-substituent may not be important with regard to interactions with the enzyme active site. Methylation of the *N*³-position, as in compounds **91** and **92**, resulted in a significant decrease in inhibitory activity, as would be expected on the basis of previously determined structure-activity requirements. The diphenyl and dibenzyl phosphate derivatives **95** and **98** were found to be relatively inactive against PARP. However, these derivatives are insoluble in aqueous media and, as such, are of limited use.

NU1025 (**37**), has been shown to potentiate the effects of a range of mechanistically diverse anticancer agents.^{264,311} The inhibitor is relatively non-toxic alone and effective in micromolar concentrations, compared to the millimolar concentrations of benzamide (**6**)/3AB (**8**) needed to achieve the same effects. These data suggest that NU1025 has the potential to be developed as a clinical agent to be used in conjunction with existing anticancer treatments. The most suitable DNA-damaging agents for the preclinical evaluation of PARP inhibitors appear to be monofunctional alkylating agents (temozolomide and MTIC), γ -irradiation, bleomycin and topoisomerase I inhibitors but not antimetabolites or topoisomerase II inhibitors.^{264,311}

Chapter Eight: Experimental

8.1	Index of Compounds Synthesised	Page No.
40	3-Methoxy-2-nitrobenzamide	162
41	3-Methoxy-2-aminobenzamide	162
43	3-Methyl-2-nitrobenzamide	163
44	3-Methyl-2-aminobenzamide	163
45	3-Methoxy-2- <i>N</i> -acetylaminobenzamide	164
46	8-Methoxy-2-methylquinazolin-4-[3 <i>H</i>]-one	164
48	2,8-Dimethylquinazolin-4-[3 <i>H</i>]-one	165
37	8-Hydroxy-2-methylquinazolin-4-[3 <i>H</i>]-one	166
49	3-Methoxy-2- <i>N</i> -benzoylaminobenzamide	166
50	3-Methyl-2- <i>N</i> -benzoylaminobenzamide	167
51	8-Methoxy-2-phenylquinazolin-4-[3 <i>H</i>]-one	167
52	8-Methyl-2-phenylquinazolin-4-[3 <i>H</i>]-one	168
53	8-Hydroxy-2-phenylquinazolin-4-[3 <i>H</i>]-one	168
55	8-Methoxy-2-(4'-nitrophenyl)quinazolin-4-[3 <i>H</i>]-one	169
57	8-Methyl-2-(4'-nitrophenyl)quinazolin-4-[3 <i>H</i>]-one	169
38	8-Hydroxy-2-(4'-nitrophenyl)quinazolin-4-[3 <i>H</i>]-one	170
59	8-Methoxy-2-(4'-trifluoromethylphenyl)quinazolin-4-[3 <i>H</i>]-one	171
61	8-Methyl-2-(4'-trifluoromethylphenyl)quinazolin-4-[3 <i>H</i>]-one	171
62	8-Hydroxy-2-(4'-trifluoromethylphenyl)quinazolin-4-[3 <i>H</i>]-one	172
63	4-Cyanobenzoyl chloride	172
65	8-Methoxy-2-(4'-cyanophenyl)quinazolin-4-[3 <i>H</i>]-one	173
66	3-Methyl-2- <i>N</i> -(4'-cyanobenzoyl)aminobenzamide	173
67	8-Methyl-2-(4'-cyanophenyl)quinazolin-4-[3 <i>H</i>]-one	174
68	8-Hydroxy-2-(4'-cyanophenyl)quinazolin-4-[3 <i>H</i>]-one	174
71	8-Methoxy-2-(4'-carboxyphenyl)quinazolin-4[3 <i>H</i>]-one	175

72	8-Methyl-2-(4'-carboxyphenyl)quinazolin-4[3 <i>H</i>]-one	175
73	8-Methoxy-2-(4'-carboxamidophenyl)quinazolin-4[3 <i>H</i>]-one	176
74	8-Methyl-2-(4'-carboxamidophenyl)quinazolin-4[3 <i>H</i>]-one	176
76	8-Methyl-2-(4'-methoxycarbonylphenyl)quinazolin-4[3 <i>H</i>]-one	177
82	3-Methoxy-2- <i>N</i> -(4'-methoxybenzoyl)aminobenzamide	178
84	8-Methoxy-2-(4'-methoxyphenyl)quinazolin-4-[3 <i>H</i>]-one	178
85	8-Methyl-2-(4'-methoxyphenyl)quinazolin-4-[3 <i>H</i>]-one	179
86	8-Hydroxy-2-(4'-hydroxyphenyl)quinazolin-4-[3 <i>H</i>]-one	179
87	8-Methyl-2-(4'-hydroxyphenyl)quinazolin-4-[3 <i>H</i>]-one	180
88	8-Methoxy-2-(4'-aminophenyl)quinazolin-4-[3 <i>H</i>]-one	180
89	8-Methyl-2-(4'-aminophenyl)quinazolin-4-[3 <i>H</i>]-one	181
90	8-Hydroxy-2-(4'-aminophenyl)quinazolin-4-[3 <i>H</i>]-one	181
91	8-Methoxy-3- <i>N</i> -methyl-2-methylquinazolin-4-[3 <i>H</i>]-one	182
92	8-Hydroxy-3- <i>N</i> -methyl-2-methylquinazolin-4-[3 <i>H</i>]-one	183
93	8- <i>O</i> -(Glycine carbamate ethyl ester)-2-methylquinazolin-4-[3 <i>H</i>]-one	183
94	8- <i>O</i> -(Glycine carbamate)-2-methylquinazolin-4-[3 <i>H</i>]-one	184
95	8-(<i>O</i> -Diphenylphosphoryl)-2-methylquinazolin-4-[3 <i>H</i>]-one	185
96	Attempted preparation of 8-(<i>O</i> -phosphoryl)-2-methylquinazolin-4-[3 <i>H</i>]-one	185
97	Dibenzyl phosphorochloridate	186
98	8-(<i>O</i> -Dibenzylphosphoryl)-2-methylquinazolin-4-[3 <i>H</i>]-one	186
96	8-Methyl-2-(4'- <i>O</i> -dibenzylphosphorylphenyl)quinazolin-4-[3 <i>H</i>]-one	187
99	8-Methyl-2-(4'- <i>O</i> -dibenzylphosphorylphenyl)quinazolin-4-[3 <i>H</i>]-one	188
104	Methyl 2-amino-3-nitrobenzoate	189
105	Methyl 2,3-diaminobenzoate	190
117	Methyl 2-amino-3- <i>N</i> -(4'-methoxybenzoyl)aminobenzoate	190
118	Methyl 2,3- <i>N, N</i> -bis-(4'-methoxybenzoyl)aminobenzoate	191
119	Methyl 2,3-diaminobenzoate hydrochloride salt	191
120	Methyl 2-(4'-methoxyphenyl)-1 <i>H</i> -benzimidazole-4-carboxylate acetate salt	192
121	2-(4'-Methoxyphenyl)-1 <i>H</i> -benzimidazole-4-carboxamide	192

109	2-(4'-Hydroxyphenyl)-1 <i>H</i> -benzimidazole-4-carboxamide	193
126	Isonitrosoacet- <i>o</i> -nitroanilide	194
127	Attempted preparation of 7-nitroisatin	194
133	2-(4'-Chlorophenyl)-1 <i>H</i> -benzimidazole-4-carboxylate	195
134	2-(4'-Chlorophenyl)-1 <i>H</i> -benzimidazole-4-carboxamide	195
135	2-(4'-Hydroxyphenyl)-1 <i>H</i> -benzimidazole-4-carboxylate	196
109	2-(4'-Hydroxyphenyl)-1 <i>H</i> -benzimidazole-4-carboxamide	196
136	2-(2'-Methoxyphenyl)-1 <i>H</i> -benzimidazole-4-carboxylate	197
137	2-(2'-Methoxyphenyl)-1 <i>H</i> -benzimidazole-4-carboxamide	197
138	2-(2'-Hydroxyphenyl)-1 <i>H</i> -benzimidazole-4-carboxylate	198
139	2-(2'-Hydroxyphenyl)-1 <i>H</i> -benzimidazole-4-carboxamide	198
140	2-(4'-Hydroxyphenyl-3'-methoxy)-1 <i>H</i> -benzimidazole-4-carboxylate	199
141	2-(4'-Hydroxyphenyl-3'-methoxy)-1 <i>H</i> -benzimidazole-4-carboxamide	199
142	2-(3',4'-Dimethoxyphenyl)-1 <i>H</i> -benzimidazole-4-carboxylate	200
143	2-(3',4'-Dimethoxyphenyl)-1 <i>H</i> -benzimidazole-4-carboxamide	200
144	2-(3',4',5'-Trimethoxyphenyl)-1 <i>H</i> -benzimidazole-4-carboxylate	201
145	2-(3',4',5'-Trimethoxyphenyl)-1 <i>H</i> -benzimidazole-4-carboxamide	201
146	2-(4'- <i>N,N</i> -Dimethylaminophenyl)-1 <i>H</i> -benzimidazole-4-carboxylate	202
147	2-(4'- <i>N,N</i> -Dimethylaminophenyl)-1 <i>H</i> -benzimidazole-4-carboxamide	202
148	2-(4'-Ethoxycarbonylphenyl)-1 <i>H</i> -benzimidazole-4-carboxylate	203
149	2-(4'-Ethoxycarbonylphenyl)-1 <i>H</i> -benzimidazole-4-carboxamide	203
150	2-(3',4'-Methylenedioxyphenyl)-1 <i>H</i> -benzimidazole-4-carboxylate	204
151	2-(3',4'-Methylenedioxyphenyl)-1 <i>H</i> -benzimidazole-4-carboxamide	204
155	<i>N,N</i> -Diisopropyl- <i>O</i> -benzylisourea	205
156	4-Benzylloxycarbonylbenzaldehyde	205
157	2-(4'- <i>O</i> -Dibenzylphosphorylphenyl)-1 <i>H</i> -benzimidazole-4-carboxamide	206
159	2-(4'- <i>O</i> -Phosphorylphenyl)-1 <i>H</i> -benzimidazole-4-carboxamide ammonium salt	207

8.2 General Experimental Details

Analytical Procedures

Melting points are uncorrected and were determined with a Kofler hot stage apparatus. Elemental combustion analyses were carried out, in house, on a Carlo-Erba Instrumentazione 1106 analyser or were performed by Butterworths Laboratories Ltd, 54-56 Waldegrave Road, Teddington, Middlesex, TW11 8LG. The figures given are within $\pm 0.4\%$ of theory unless otherwise specified.

Infrared spectra were recorded using samples mounted in potassium bromide discs, on a Nicolet 20 PC Fourier Transform spectrometer.

Proton (^1H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded at 200 MHz and 50 MHz, respectively, on a Bruker WP 200 Spectrometer, employing tetramethylsilane (TMS) as internal standard. Spectra were obtained in deuteriated dimethylsulphoxide (d_6 -DMSO) and exchangeable protons were 'removed' using a deuterium oxide (D_2O) 'shake'. Abbreviations for multiplicity of peaks in spectra include: s (singlet), d (doublet), dd (double-doublet), t (triplet), q (quartet), m (multiplet), br (broad) and combinations thereof. Chemical shift values are quoted in parts per million (ppm). Coupling constants (J) are given in Hertz (Hz). ^1H NMR spectra of *para*-disubstituted aromatic rings were observed as the required AA'BB' pattern and are quoted, for simplicity, as doublets.

Mass spectra were recorded using either electron impact (EI) or fast atom bombardment (FAB) techniques (see main text), on a Kratos MS80 RF Spectrometer. FAB spectra were obtained using a *meta*-nitrobenzyl alcohol matrix.

Reagents and Solvents

Reagents were purchased from Aldrich Chemical Company, Fluka Chemie AG or Lancaster Synthesis Ltd. Where appropriate, experimental procedures were carried out under a positive pressure of nitrogen. Ethyl acetate (EtOAc), dichloromethane (DCM) and petrol (in the boiling range 40-60 °C) were redistilled prior to use. Dry solvents were prepared as required:³¹⁴ Tetrahydrofuran (THF) and ether were distilled from sodium/benzophenone and

used immediately. Methanol (MeOH) and ethanol (EtOH) were dried using magnesium/iodine and distilled. Acetonitrile (MeCN) and triethylamine (NEt₃) were pre-dried with calcium hydride (CaH₂) and distilled. Pyridine was distilled from CaH₂ and stored over potassium hydroxide pellets. *N,N*-diisopropylethylamine (Hünig's base) was distilled from ninhydrin. Unless otherwise stated, dry solvents were stored until required over 3Å or 4Å molecular sieves under nitrogen.

Chromatography

Thin layer chromatography (TLC) was carried out using Merck 1.05554 aluminium plates, pre-coated with silica gel 60F₂₅₄, and a variety of mobile phases. TLC plates were visualised using short wave (254 nm) and long wave (365 nm) ultraviolet light. Silica gel (Fison's Matrix Silica 60) was used for column chromatography procedures, which were carried out either at medium pressure or under gravity (see main text for solvent systems).

Pressure Reaction

A sealed pressure vessel was required for the reaction involving liquid ammonia at 40 atm. The vessel was designed and built in the mechanical engineering workshops in the Department of Chemistry, University of Newcastle upon Tyne.

8.3 Standard Procedures

For convenience, standard procedures have been given, as similar procedures were employed for important reactions concerning the synthesis of precursors and derivatives of the quinazolin-4-[3*H*]-ones and 2-arylbenzimidazoles. Variations from these procedures and purification methods are given in the main text.

Standard Procedure A: *Preparation of 3-Substituted-2-N-Substituted Aminobenzamides.*

The appropriate 3-substituted-2-aminobenzamide (1 equivalent, 3.0-6.7 mmol) was dissolved in dry THF with dry pyridine (1.3 equivalents) and stirred under nitrogen at room temperature.

The required acid chloride (1-1.5 equivalents) was dissolved in dry THF and added dropwise to the reaction mixture, which was stirred at room temperature until TLC indicated the absence of starting material (further equivalents of acid chloride were added to achieve this in some cases). The solvents were removed under reduced pressure and the remaining white slurry was filtered and washed with sodium bicarbonate solution and water. The product was purified by column chromatography and/or recrystallisation from suitable solvents.

Standard Procedure B: *Quinazolinone Ring Formation under Basic Conditions.*

The starting material (0.4-1.6 mmol) was suspended in aq. NaOH solution of appropriate strength and stirred until complete dissolution was obtained (with warming, if necessary). The solution was neutralised (pH 7) by careful addition of conc. HCl and the resulting precipitate was collected by filtration and washed with water. The product was purified by recrystallisation from suitable solvents.

Standard Procedure C: *Preparation of Substituted Quinazolin-4-[3H]-ones from 3-Substituted-2-aminobenzamides.*

The appropriate 3-substituted-2-aminobenzamide (1 equivalent, 1.2-9.0 mmol) was dissolved in dry THF. Dry pyridine (1.3 equivalents) was added and the solution was stirred under nitrogen at room temperature. The required acid chloride (1-1.5 equivalents) was dissolved in dry THF and added dropwise to the reaction mixture, which was stirred at room temperature until TLC indicated the absence of starting material (further equivalents of acid chloride were added to achieve this in some cases). The solvents were removed under reduced pressure and the remaining residue was resuspended in aq. NaOH solution of appropriate strength and stirred until complete dissolution was obtained (with warming, if necessary). The solution was neutralised (pH 7) by careful addition of conc. HCl and the resulting precipitate was collected by filtration and washed with water. The product was purified by column chromatography and/or recrystallisation from a suitable solvent.

Standard Procedure D: Lewis Acid-Catalysed Demethylation of 8-Methoxy-2-substituted Quinazolin-4-[3H]-one Derivatives.²⁶⁶

The appropriate 8-methoxy-2-substituted quinazolin-4-[3H]-one (1 equivalent, 0.3-3.7 mmol) was suspended in a 1.0 M solution of BBr₃ in DCM (1 equivalent/methoxy group + 1 equivalent/atom bearing lone pairs) and stirred under gentle reflux for up to 48 hours. Once TLC indicated the absence of starting material, the solvent was directly distilled from the reaction vessel and the solid residue was *cautiously* redissolved in 10% aq. NaOH solution. After at least 2 hours, the solution was neutralised (pH 7) by the careful addition of conc. HCl. The product was extracted into ethyl acetate (EtOAc) and washed with water: in some cases it was possible to collect most of the product by filtration after neutralisation, and the remaining aq. layer was extracted with EtOAc. The organic layer was dried (Na₂SO₄), filtered and the product was obtained by removal of the solvent under reduced pressure. The product was purified by column chromatography and/or recrystallisation from suitable solvents.

Standard Procedure E: Preparation of 2-Substituted Benzimidazole-4-Carboxylic Acids.³⁰³

A solution of 3-nitroanthranilic acid (1 equivalent, 4.1-27.0 mmol)) and NaOH (1.1 equivalents) in water was hydrogenated (Pd/C/H₂) until no further hydrogen uptake was observed. The catalyst was removed by filtration through Celite and the filtrate was acidified (pH 4-5) with glacial acetic acid. A solution of the appropriate aldehyde (1.4 equivalents) in methanol was added, followed by a solution of cupric acetate (1.4 equivalents) in water. The resulting mixture was stirred vigorously, heated briefly to boiling and filtered hot. The precipitate was washed with water and dissolved in ethanol containing conc. HCl (1 ml HCl/20 ml ethanol). A solution of Na₂S* (1.4 equivalents) in water was added (keeping the solution acidic by addition of more HCl if necessary), and the mixture was filtered hot through Celite, to remove the copper sulfide. The pH of the filtrate was adjusted to 5-6 and diluted with water. The ethanol was removed under reduced pressure and the pH of the aq. layer was adjusted to 4-5. The crude product was collected by filtration, and washed with water. The

* Extreme Caution: Na₂S in acidic media generates the highly toxic gas H₂S.

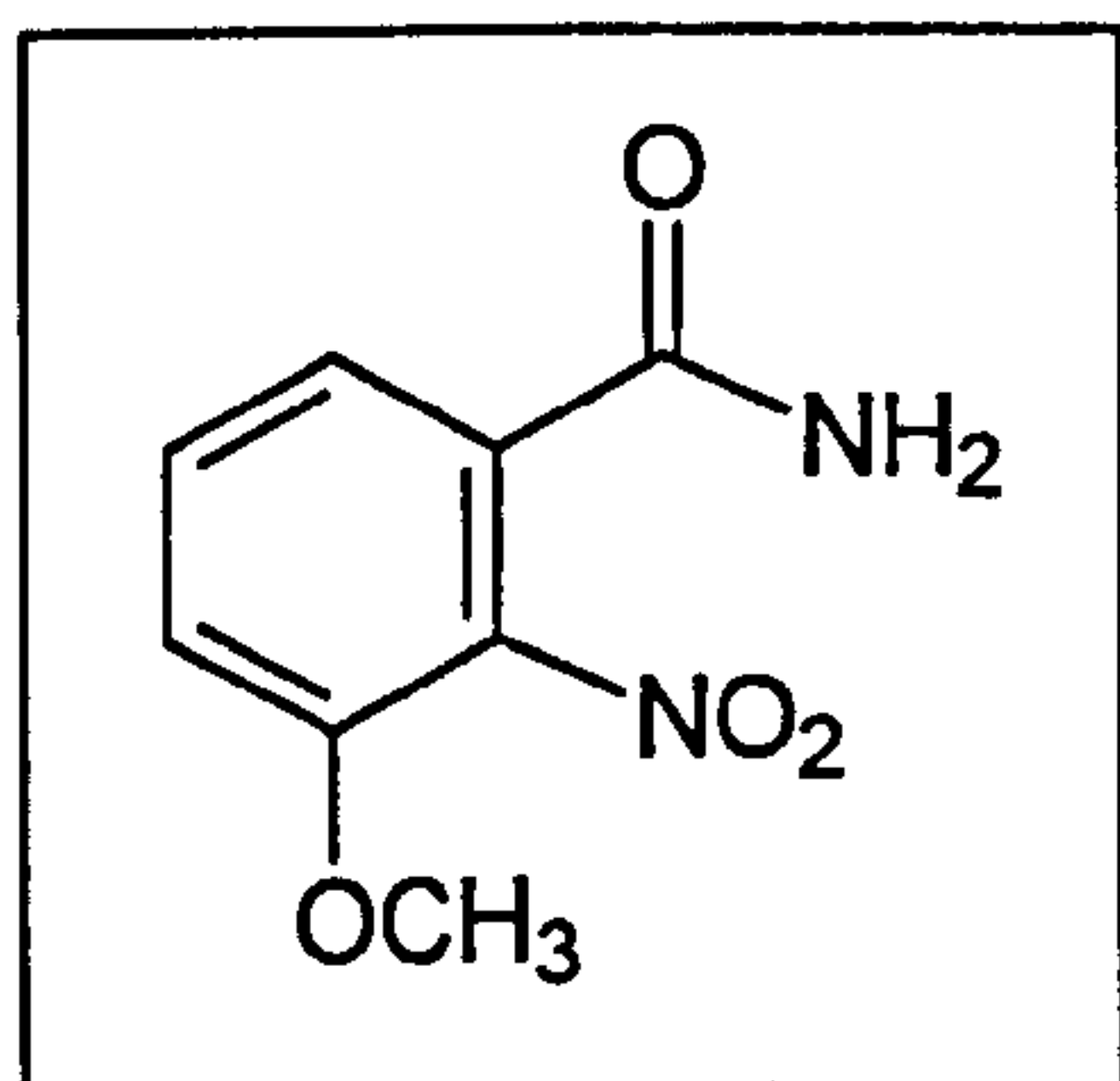
crude product was dissolved in 0.5 M NaOH solution and extracted with EtOAc. The pH of the aq. layer was adjusted to 4-5 and the precipitate was collected by filtration and washed with water. The product was either purified by column chromatography and/or recrystallisation from suitable solvents, or used crude.

Standard Procedure F: *Synthesis of 2-Aryl Benzimidazole-4-Carboxamides.*

The appropriate benzimidazole-4-carboxylate (1 equivalent, 0.4-3.9 mmol) was suspended in dry THF. Thionyl chloride (1.5 equivalents) and 2 drops of dry DMF were added and the reaction mixture was stirred at room temperature and monitored by TLC (adding further equivalents of thionyl chloride if necessary). Once TLC indicated the absence of starting material, the reaction mixture was added dropwise to an excess of stirred aq. ammonia solution. After 30 minutes, the volatiles were removed under reduced pressure and the remaining aq. slurry was filtered to give the crude product, which was washed thoroughly with water. The product was purified by column chromatography and/or recrystallisation from suitable solvents.

8.4 Quinazolinone Experimental Procedure

40 3-Methoxy-2-nitrobenzamide

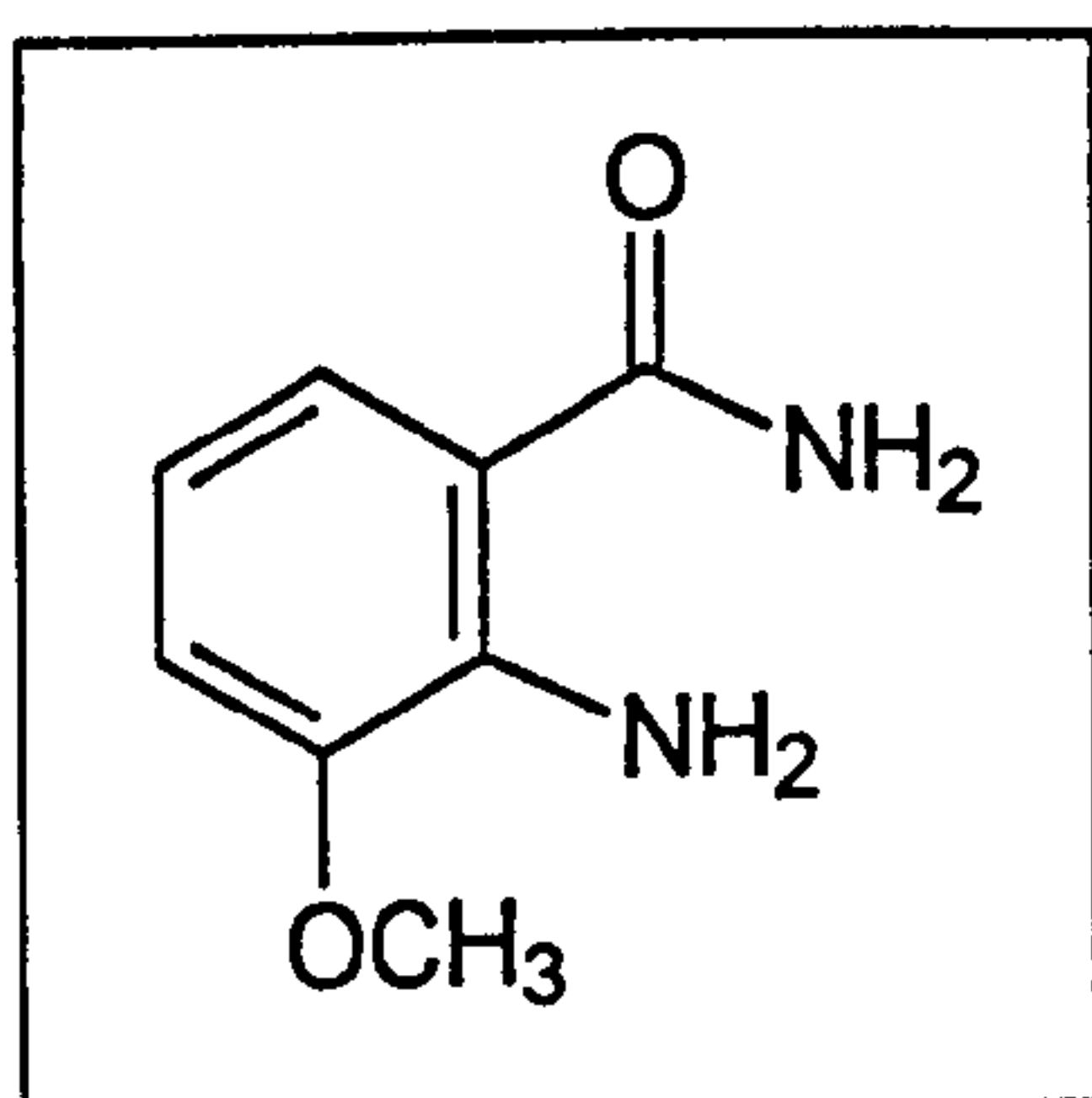


3-Methoxy-2-nitrobenzoic acid **39** (3.0 g, 15 mmol) was dissolved in dry THF (50 ml). Thionyl chloride (1.7 ml, 23 mmol) was added, with 2 drops of DMF and the reaction mixture was stirred for 12 hours under nitrogen at room temperature. The reaction mixture was added dropwise to an excess of conc. ammonia

solution (18 ml) and a cream precipitate formed. After 15 minutes, most of the solvent was removed under vacuum. The remaining slurry was filtered and the cream product was washed with ice-cold water (2.9 g, 15 mmol, 97%), m.p. 219-222 °C (lit. m.p.²⁶⁵ 212.5-213.0 °C).

Anal. found C 49.03, H 3.93, N 13.97, $C_8H_8N_2O_4$ requires C 48.98, H 4.11, N 14.28%; $\nu_{\max}/\text{cm}^{-1}$ 3347 and 3179 (br, 1° amide NH), 2802 (OCH_3), 1672 ($\text{C}=\text{O}$), 1535 (NO_2); δ_{H} (200 MHz, d_6 -DMSO) 4.01 (s, 3H, $-\text{OCH}_3$), 7.41-7.46 (dd, 1H, Ar-4H, $J = 1.2, 7.6$), 7.55-7.60 (dd, 1H, Ar-6H, $J = 1.1, 8.5$), 7.69-7.77 (t, 1H, Ar-5H, $J = 7.7$), 7.84 (s, 1H, $-\text{NH}$), 8.31 (s, 1H, $-\text{NH}$); δ_{C} (d_6 -DMSO) 57.15 ($-\text{OCH}_3$), 116.21, 119.99 (Ar-4/5C), 129.83 (Ar-1C), 131.79 (Ar-6C), 150.78 (Ar-2C), 165.82 ($\text{C}=\text{O}$); m/z (EI) 196 (M^+ , 100%), 180 ($[\text{M}-\text{O}]^+$), 149 ($[\text{M}-\text{OCH}_3\text{O}]^+$).

41 3-Methoxy-2-aminobenzamide



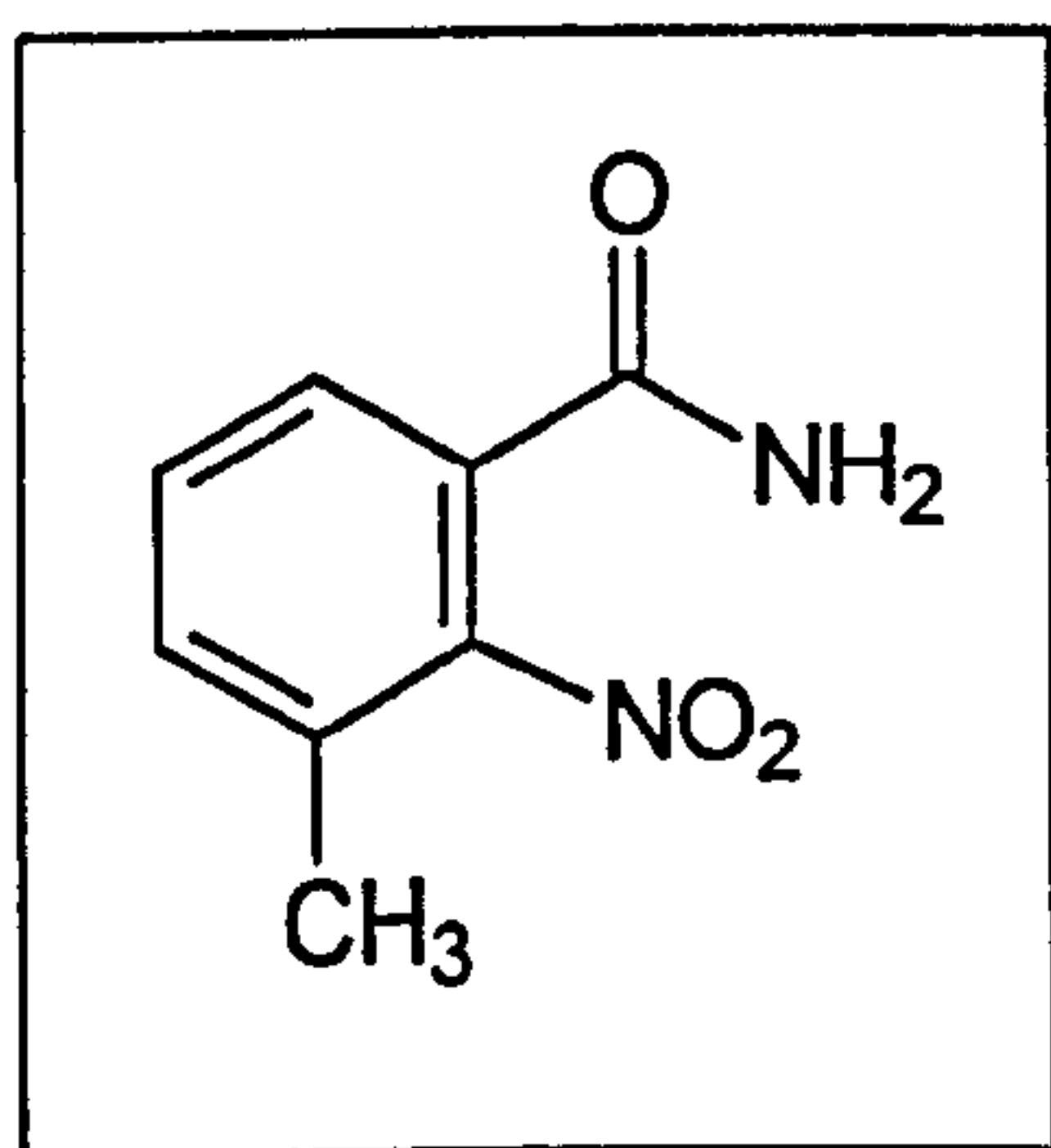
3-Methoxy-2-nitrobenzamide **40** (1.4 g, 7.1 mmol) was dissolved in dry methanol (80 ml) containing suspended 10% palladium-carbon catalyst (150 mg). The reaction vessel was placed under an atmosphere of hydrogen, at ambient temperature and pressure, until no further hydrogen absorption was observed. The catalyst was

removed by filtration through Celite to leave a colourless solution. The solvent was removed

under vacuum to afford the title compound as a pale pink solid (1.2 g, 7.0 mmol, 99%), m.p. 145-147 °C (lit. m.p.²⁶³ 146-147 °C).

Anal. found C 57.54, H 5.99, N 16.61, $C_8H_{10}N_2O_2$ requires C 57.82, H 6.07, N 16.86%; $\nu_{\max}/\text{cm}^{-1}$ 3473 (NH), 3365 (NH), 3332 and 3149 (br, 1° amide NH), 2832 (OCH₃), 1669 and 1617 (C=O); δ_H (200 MHz, d_6 -DMSO) 3.88 (s, 3H, -OCH₃), 6.40 (s, 2H, -NH₂), 6.54-6.62 (t, 1H, Ar-5H, $J = 8.0$), 6.96-6.99 (dd, 1H, Ar-4H, $J = 7.8$), 7.23 (s, 1H, -NH), 7.29-7.33 (dd, 1H, Ar-6H, $J = 1.0, 7.2$), 7.85 (s, 1H, -NH); m/z (EI) 166 (M^+ , 100%), 149 ($[M-NH_3]^+$), 134 ($[M-NH_2OCH_3]^+$).

43 3-Methyl-2-nitrobenzamide



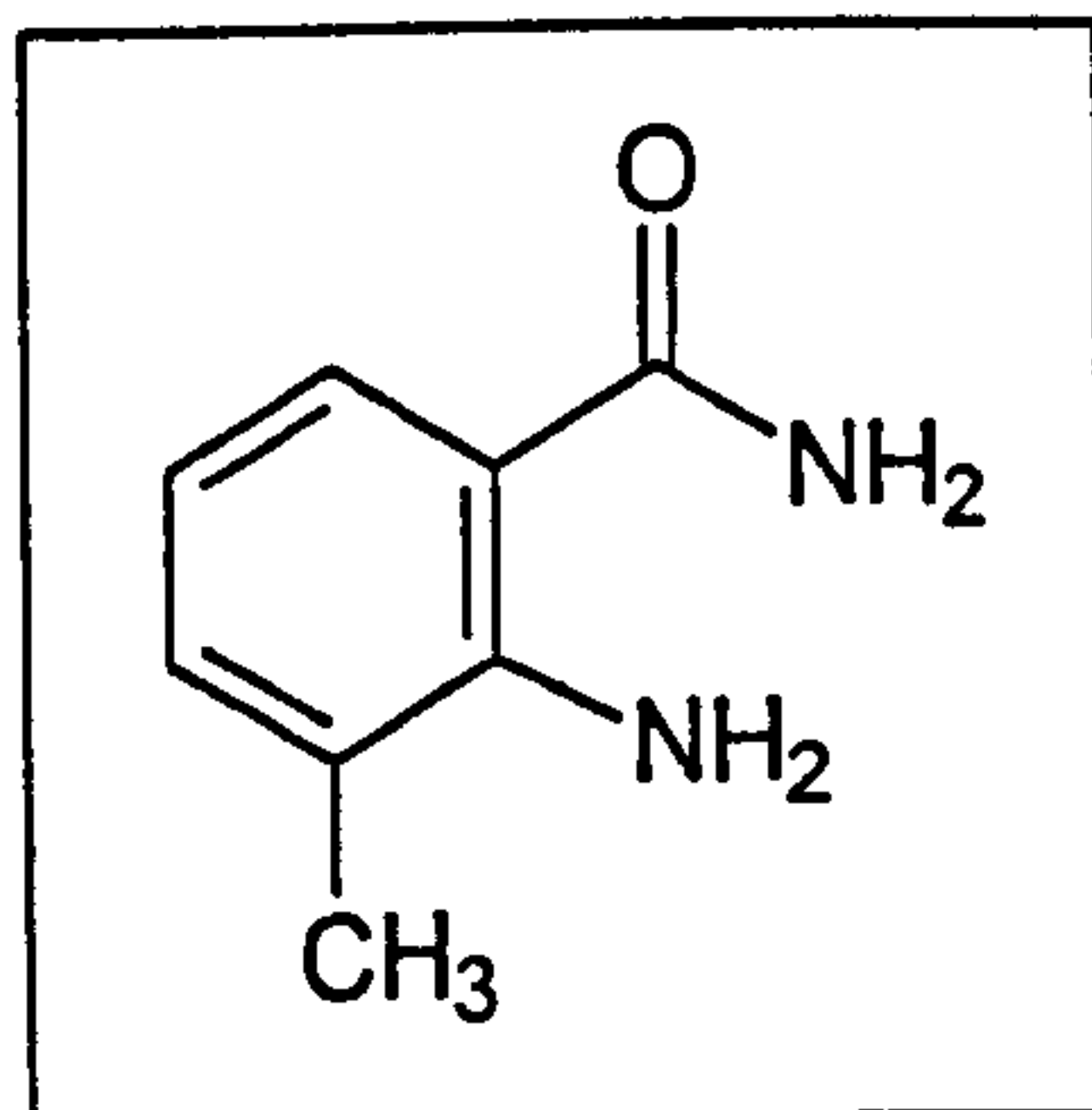
Prepared in a similar way to compound 40 from 3-methyl-2-nitrobenzoic acid 42 (2.0 g, 11.0 mmol) to give the product as a cream solid.

Yield : 1.9 g, 10.0 mmol, 95%, m.p. 198-199 °C.

Anal. found C 53.28, H 4.30, N 15.25, $C_8H_8N_2O_3$ requires C 53.33, H 4.48, N 15.55%; $\nu_{\max}/\text{cm}^{-1}$ 3372 and 3179 (br, 1° amide NH),

2969, 2931 and 2879 (CH₃), 1651 and 1620 (C=O), 1527 (NO₂); δ_H (200 MHz, d_6 -DMSO) 2.37 (s, 3H, -CH₃), 7.66 (s, 3H, Ar-4/5/6H), 7.79 (s, 1H, -NH), 8.30 (s, 1H, -NH); m/z (EI) 180 (M^+ , 100%), 164 ($[M-O]^+$), 77 (Ph^+).

44 3-Methyl-2-aminobenzamide



Prepared in a similar way to compound 41 from 3-methyl-2-nitrobenzamide 43 (1.2 g, 6.7 mmol) to give the product as a cream solid.

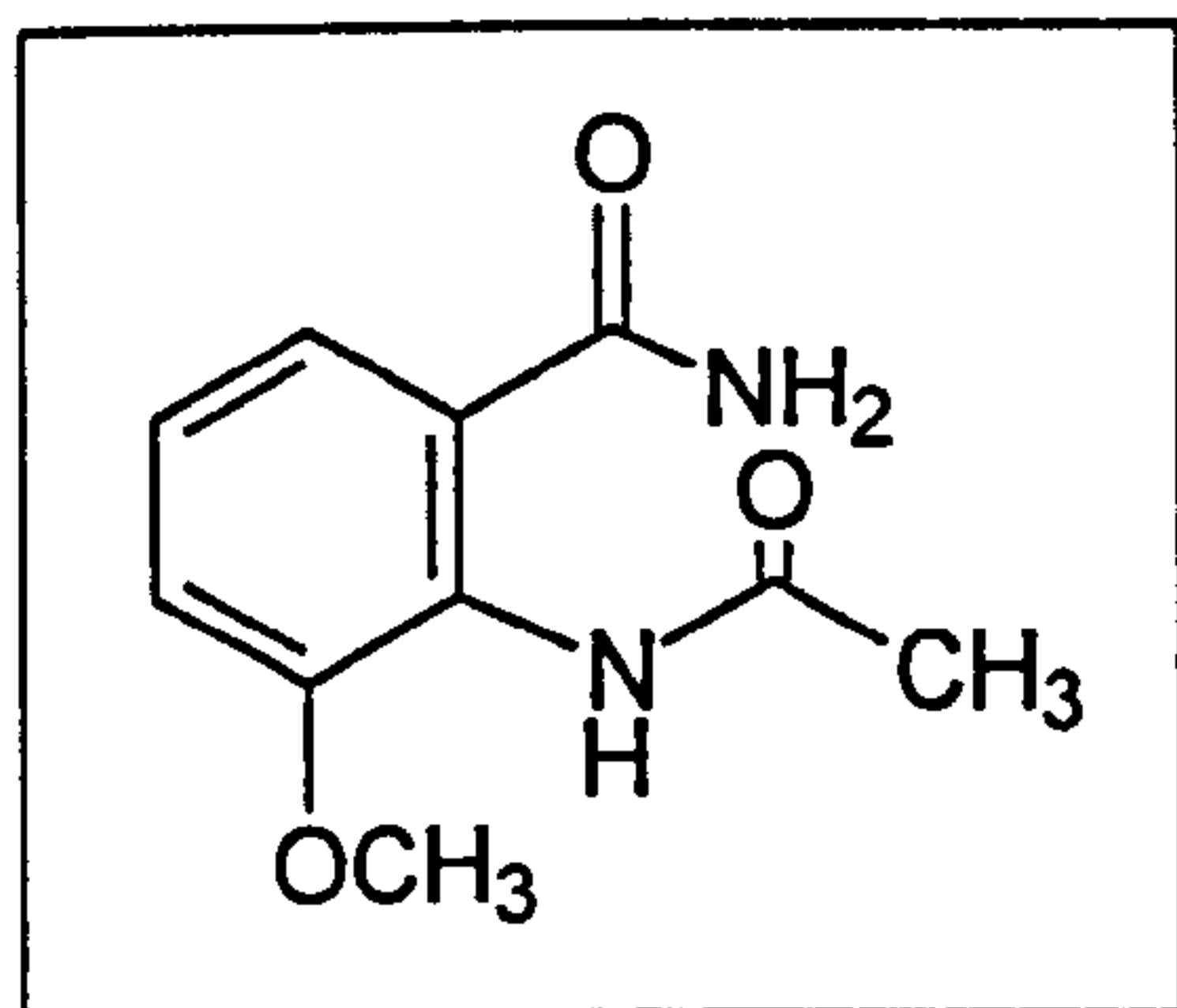
Yield : 0.97 g, 6.4 mmol, 97%, m.p. 145-147 °C.

Anal. found C 63.36, H 6.55, N 18.20, $C_8H_{10}N_2O$ requires C 63.98,

H 6.71, N 18.66%; $\nu_{\max}/\text{cm}^{-1}$ 3468 (NH), 3391 and 3190 (br, 1° amide NH), 2981 (CH₃), 1641

and 1608 (C=O); δ_{H} (200 MHz, d_6 -DMSO) 2.18 (s, 3H, -CH₃), 6.52-6.59 (t, 1H, Ar-5H, J = 6.6, 7.7), 7.15-7.19 (d, 1H, Ar-4H, J = 7.0), 7.51-7.55 (d, 1H, Ar-6H, J = 7.8), 7.85 (s, 1H, -NH); m/z (EI) 150 (M^+ , 55%), 133 ($[M-\text{NH}_3]^+$), 105 ($\text{PhC}=\text{O}^+$), 77 (Ph^+).

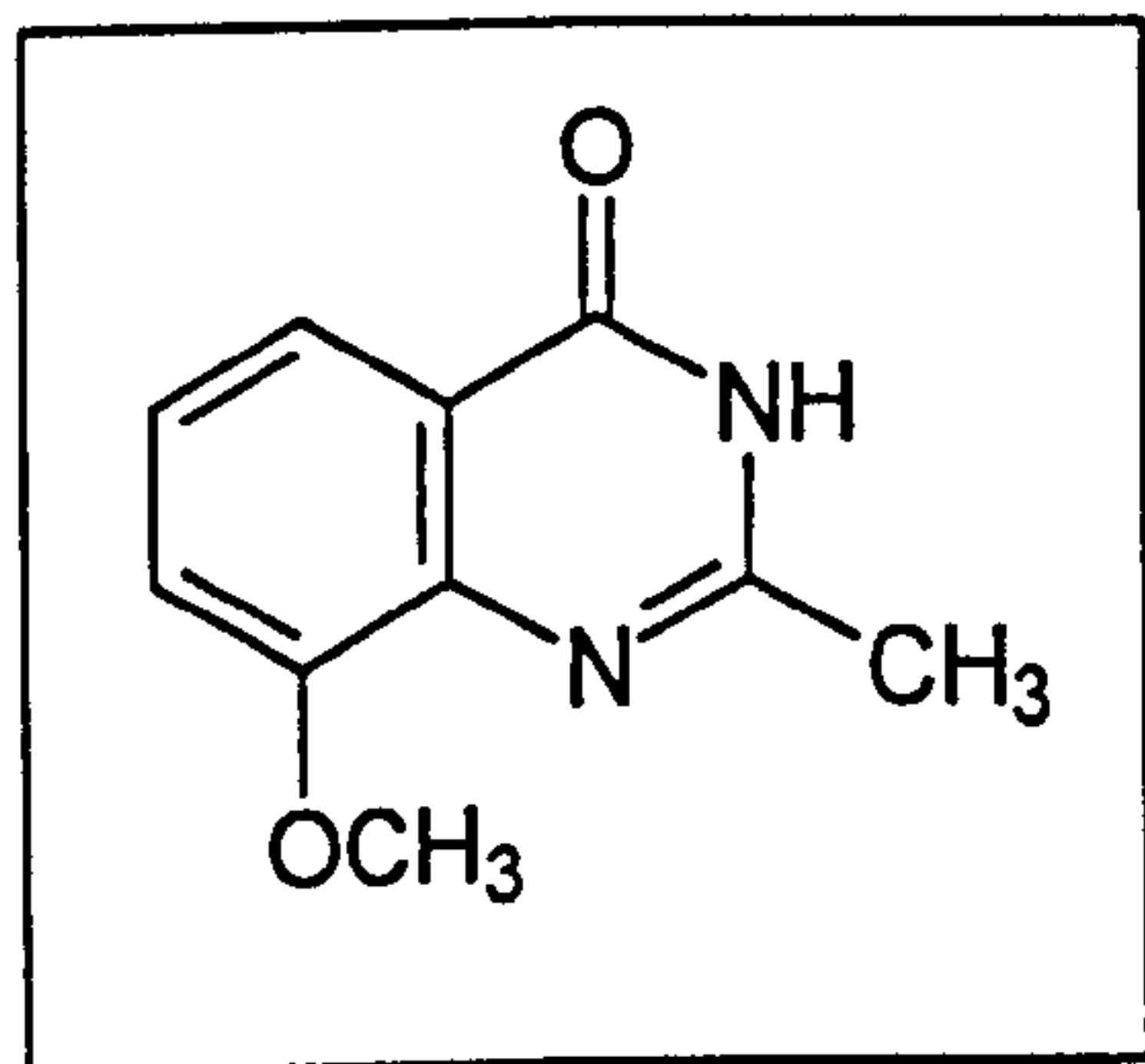
45 3-Methoxy-2-*N*-acetyl aminobenzamide



Prepared according to **Standard Procedure A** from 3-methoxy-2-aminobenzamide **41** (0.5 g, 3.0 mmol) and acetyl chloride (0.2 ml, 3.3 mmol). The product was recrystallised from methanol/water to give a white crystalline solid (0.19 g, 0.9 mmol, 31%), m.p. 243-246 °C.

Anal. found C 56.98, H 5.38, N 12.78, $\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}_3 \cdot 0.15 \text{ mol. H}_2\text{O}$ requires C 56.94, H 5.88, N 13.28%; $\nu_{\text{max}}/\text{cm}^{-1}$ 3424 (NH), 3249 and 3160 (br, 1° amide NH), 2970 (CH₃), 2844 (OCH₃), 1660 (C=O); δ_{H} (200 MHz, d_6 -DMSO) 2.05 (s, 3H, -CH₃), 3.88 (s, 3H, -OCH₃), 7.14-7.18 (dd, 1H, Ar-4H, J = 1.4, 7.5), 7.21-7.25 (dd, 1H, Ar-6H, J = 1.4, 7.6), 7.33-7.41 (m, 2H, -NH and Ar-5H), 7.53 (s, 1H, -NH), 9.27 (s, 1H, -NH); m/z (EI) 208 (M^+ , 17%), 190 ($[M-\text{H}_2\text{O}]^+$), 166 ($[M-\text{COCH}_2]^+$).

46 8-Methoxy-2-methylquinazolin-4-[3H]-one



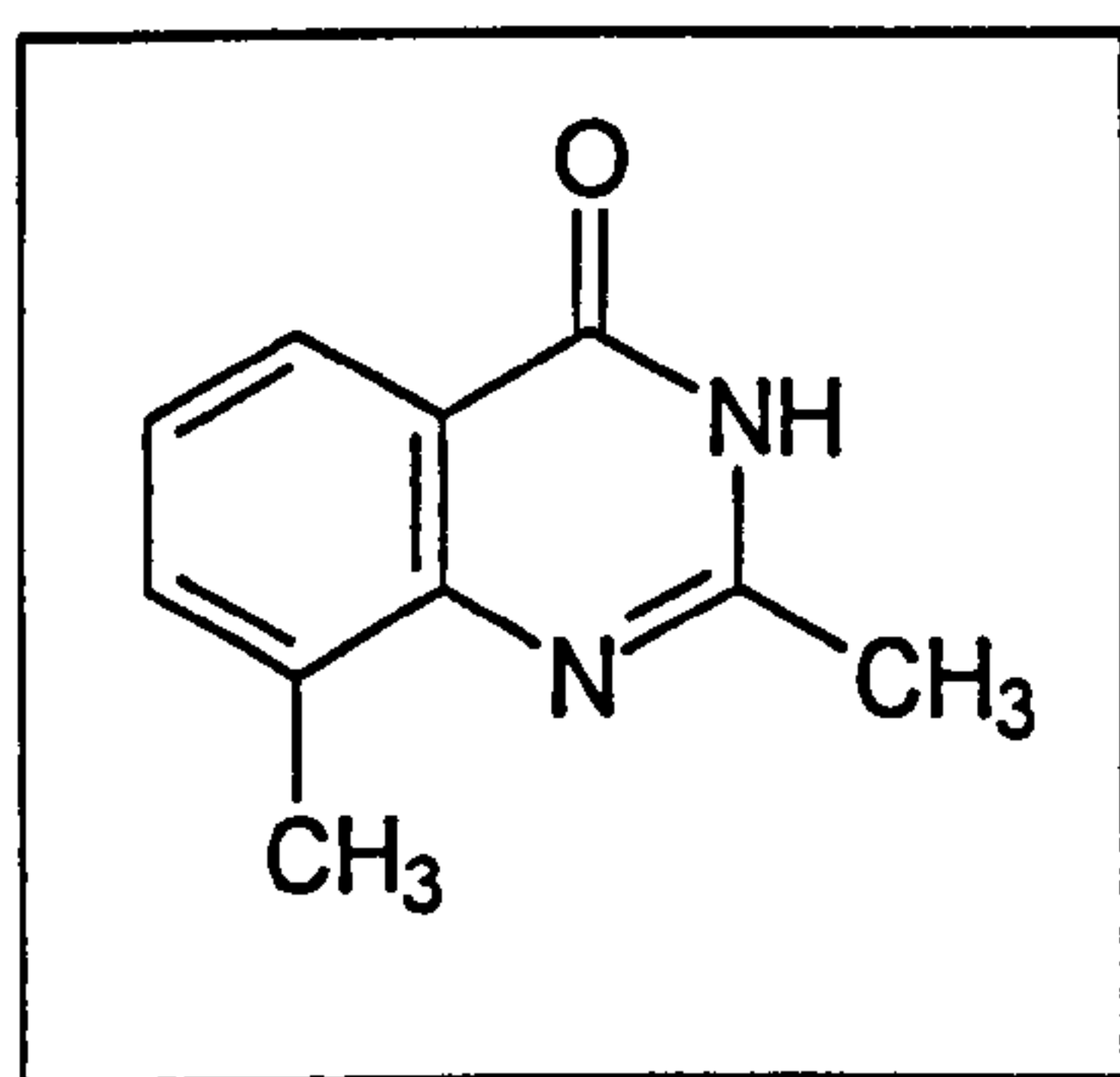
A. Prepared according to **Standard Procedure B** from 3-methoxy-2-*N*-acetyl aminobenzamide **45** (0.07 g, 0.34 mmol), in 2% aq. NaOH solution (2 ml) at room temperature (2 hours). The crude product was recrystallised from the minimum amount of boiling ethyl acetate to give white crystals (0.04 g, 0.22 mmol, 67%), m.p. 202-204 °C (sublimation).

B. Prepared according to **Standard Procedure C** from 3-methoxy-2-aminobenzamide **41** (1.5 g, 9.0 mmol) and acetyl chloride (1.4 ml, 19.9 mmol). The product was recrystallised

from methanol/water to give white crystals (1.67 g, 8.8 mmol, 97%), m.p. 202-204 °C (sublimation).

Anal. found C 62.36, H 5.29, N 14.36, $C_{10}H_{10}N_2O_2 \cdot 0.1 \text{ mol. H}_2O$ requires C 62.55, H 5.25, N 14.59%; $\nu_{\max}/\text{cm}^{-1}$ 3171 and 3034 (2° amide NH), 2903 (CH_3), 2793 (OCH_3), 1676 ($\text{C}=\text{O}$); δ_{H} (200 MHz, d_6 -DMSO) 2.43 (s, 3H, $-\text{CH}_3$), 3.97 (s, 3H, $-\text{OCH}_3$), 7.37-7.41 (dd, 1H, Ar-7H, $J = 1.9, 8.0$), 7.42-7.50 (t, 1H, Ar-6H, $J = 7.8$), 7.68-7.73 (dd, 1H, Ar-5H, $J = 1.9, 7.6$); δ_{C} (d_6 -DMSO) 21.83 ($-\text{CH}_3$), 56.05 ($-\text{OCH}_3$), 114.96, 116.99 (Ar-6/7C), 121.95 ($\text{C}-\text{CH}_3$), 126.5 (Ar-5C), 140.0 (Ar-8aC), 153.26 (Ar-8C), 154.33 (Ar-4aC), 162.04 ($\text{C}=\text{O}$); m/z (EI) 190 (M^+ , 96.6%), 189 ($[\text{M}-\text{H}]^+$), 160 ($[\text{M}-\text{OCH}_2]^+$).

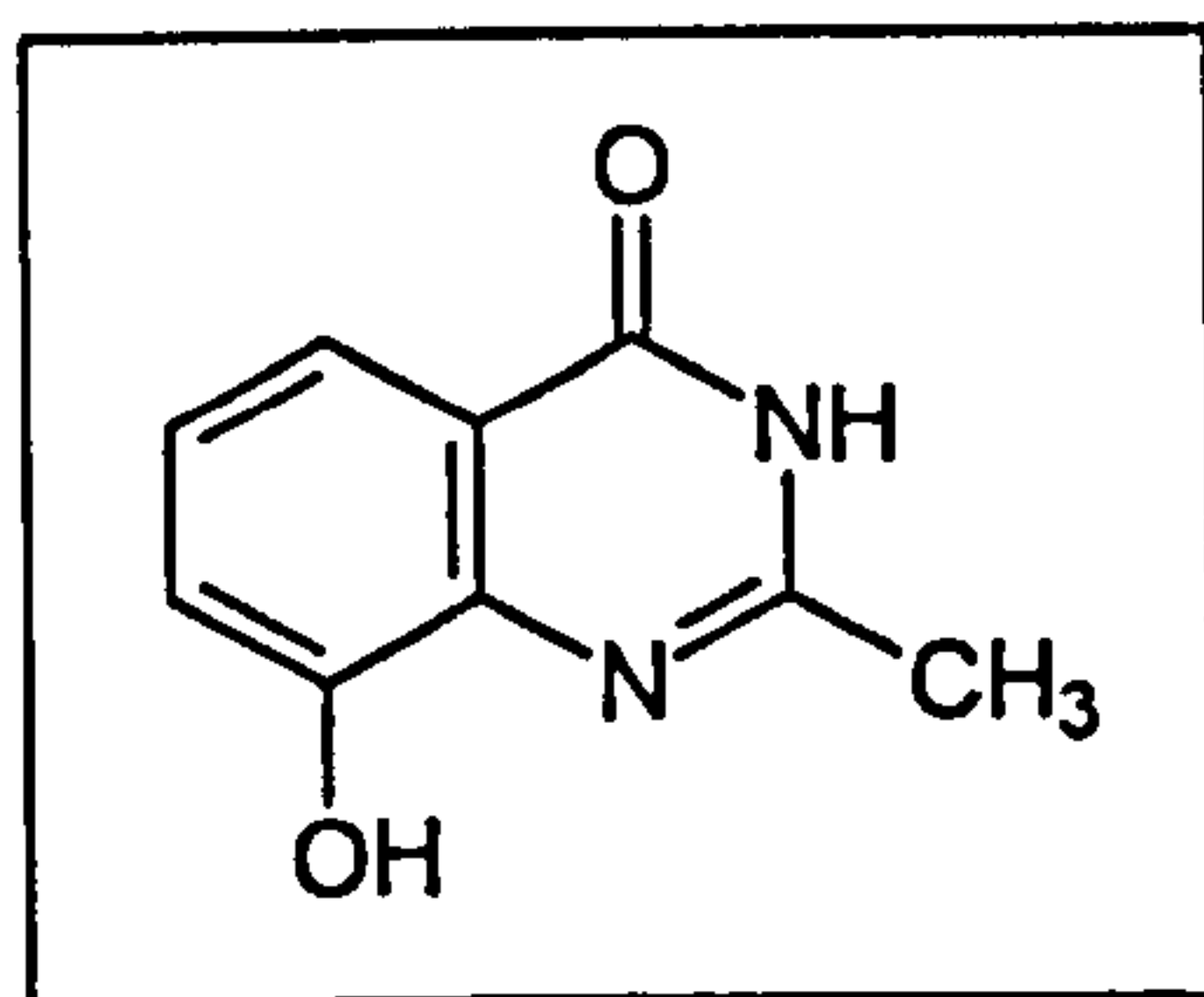
48 2,8-Dimethylquinazolin-4-[3H]-one



Prepared according to Standard Procedure C from 3-methyl-2-aminobenzamide 44 (0.5 g, 3.3 mmol) and acetyl chloride (0.36 ml, 5.0 mmol). The product was isolated as white crystals after recrystallisation from methanol/water (0.47 g, 2.7 mmol, 81%), m.p. 217-220 °C.

Anal. found C 68.76, H 5.57, N 15.90, $C_{10}H_{10}N_2O$ requires C 68.94, H 5.79, N 16.08%; $\nu_{\max}/\text{cm}^{-1}$ 3171 (2° amide NH), 2905 and 2871 (CH_3), 1683 ($\text{C}=\text{O}$); δ_{H} (200 MHz, d_6 -DMSO) 2.44 (s, 3H, $-\text{CH}_3$), 2.57 (s, 3H, $-\text{CH}_3$), 7.36-7.44 (t, 1H, Ar-6H, $J = 7.6$), 7.68-7.72 (dd, 1H, Ar-7H, $J = 0.7, 7.3$), 7.97-8.01 (dd, 1H, Ar-5H, $J = 0.7, 7.4$), 12.25 (s, 1H, $-\text{NH}$); m/z (EI) 174 (M^+ , 100%), 143 ($[\text{M}-\text{CH}_3\text{CH}_3\text{H}]^+$), 77 (Ph^+).

37 8-Hydroxy-2-methylquinazolin-4-[3H]-one

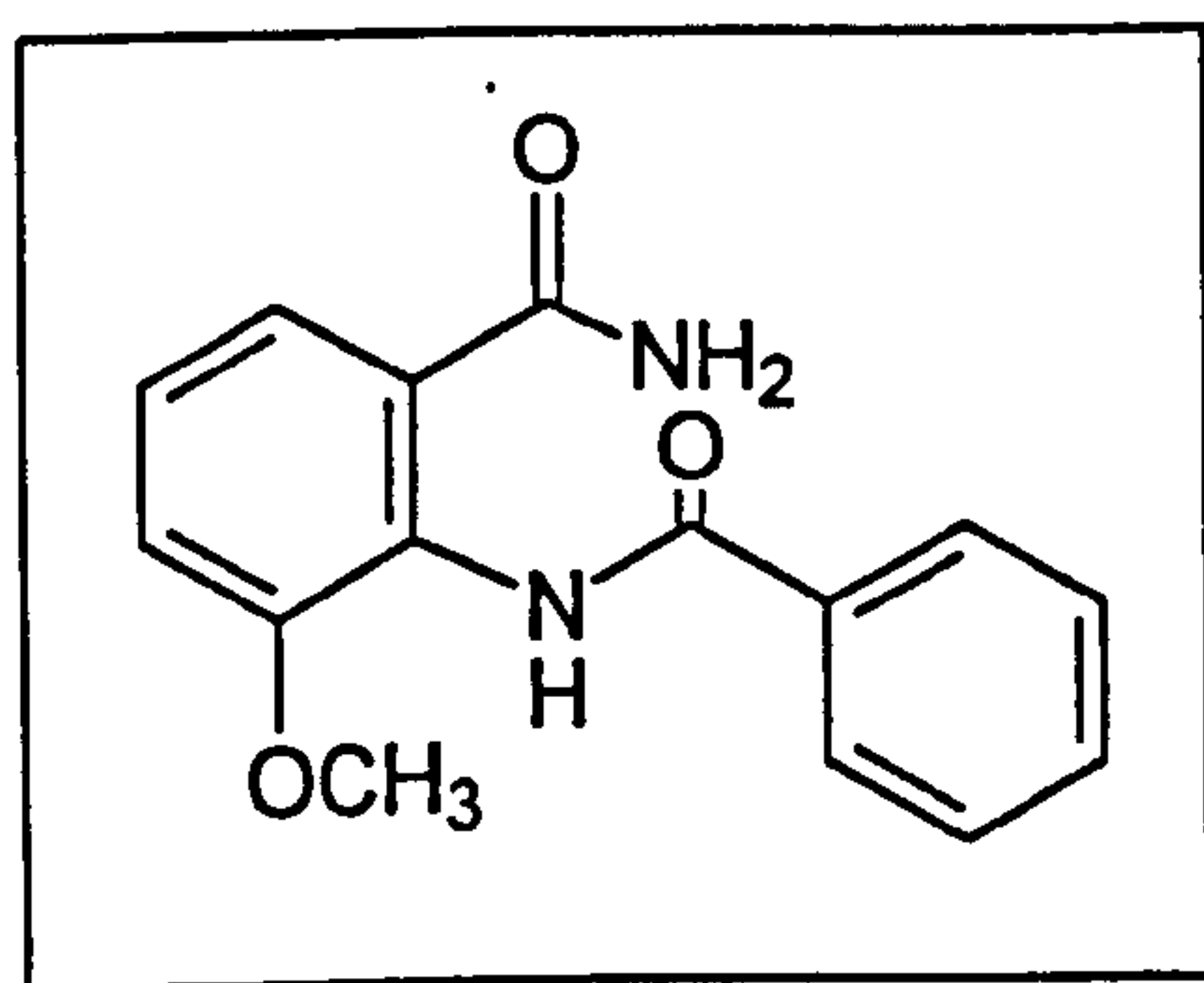


Prepared according to Standard Procedure D from 8-methoxy-2-methylquinazolin-4-[3H]-one 46 (0.7 g, 3.7 mmol). The product was recrystallised from propan-2-ol/water to give pale purple crystals (0.42 g, 2.4 mmol, 65%), m.p. 253-258 °C.

Anal. found C 61.39, H 4.54, N 15.88, $C_9H_8N_2O_2$ requires C 61.36,

H 4.58, N 15.91%; $\nu_{\max}/\text{cm}^{-1}$ 3320 (OH), 3175 and 3142 (2° amide NH), 2909 (CH_3), 1671 (C=O); δ_{H} (200 MHz, d_6 -DMSO) 2.48 (s, 3H, $-\text{CH}_3$), 7.22-7.27 (dd, 2H, Ar-7H, $J = 1.5, 7.8$), 7.28-7.41 (t, 1H, Ar-6H, $J = 7.8$), 7.57-7.63 (dd, 1H, Ar-5H, $J = 1.5, 7.8$), 9.57 (s, 1H, -OH), 12.26 (s, 1H, -NH); δ_{C} (d_6 -DMSO) 21.72 ($-\text{CH}_3$), 115.78, 118.42 (Ar-6/7C), 121.76 (C- CH_3), 126.54 (Ar-5C), 138.27 (Ar-8aC), 152.58 (Ar-8C), 152.87 (Ar-4aC), 162.05 (C=O); m/z (EI) 176 (M^+ , 100%), 163 ($[\text{M}-\text{CH}]^+$), 133 ($[\text{M}-\text{NHCHCH}_3]^+$).

49 3-Methoxy-2-N-benzoylaminobenzamide

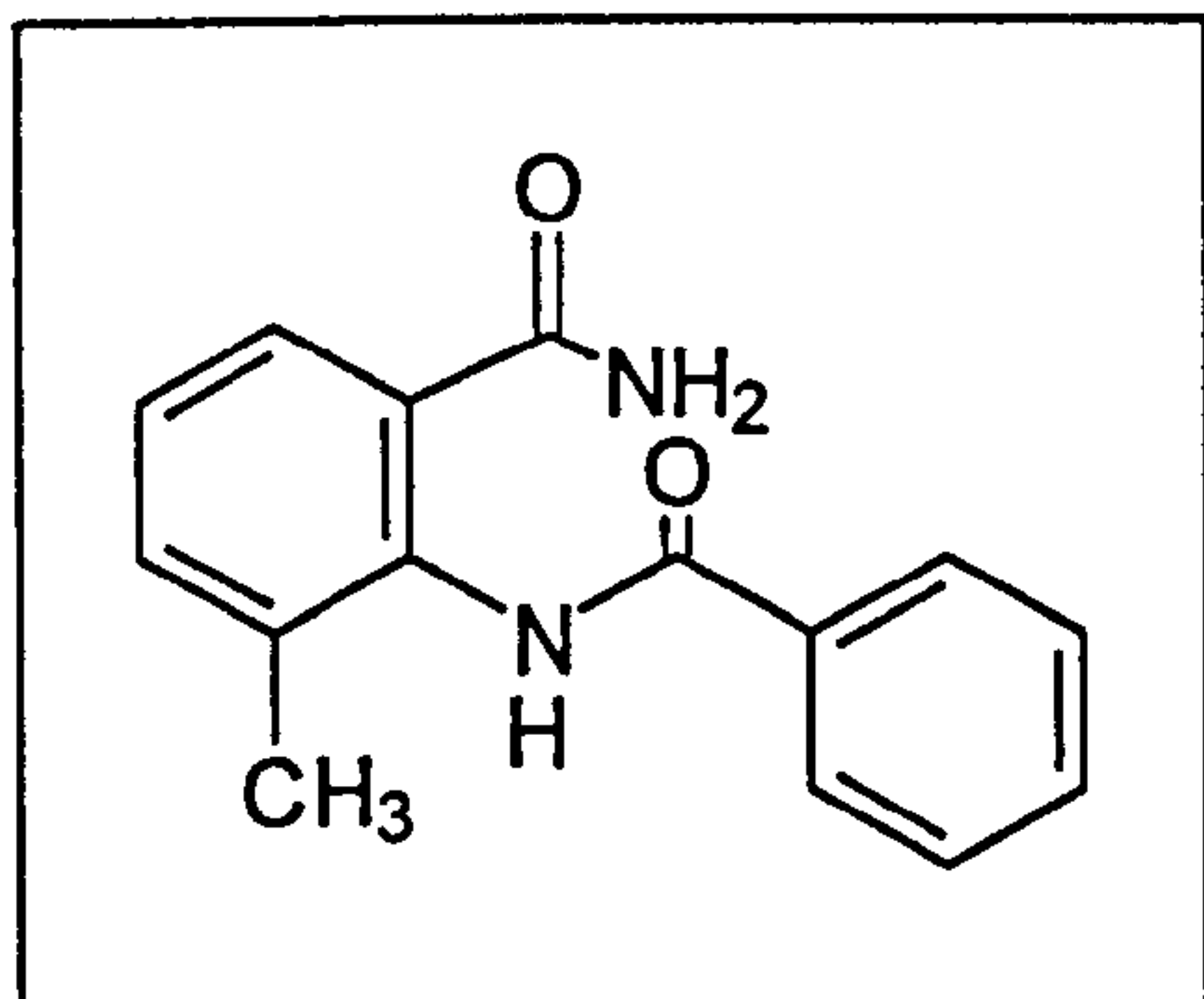


Prepared according to Standard Procedure A from 3-methoxy-2-aminobenzamide 41 (0.5 g, 3.0 mmol) and benzoyl chloride (0.4 ml, 3.3 mmol). The product (0.25 g) was recrystallised from methanol/water to give white crystals (0.20 g, 0.75 mmol, 41%), m.p. 176-180 °C.

Anal. found C 65.75, H 5.07, N 9.67, $C_{15}H_{14}N_2O_3 \cdot 0.2 \text{ mol.}$

H_2O requires C 65.76, H 5.30, N 10.23%; $\nu_{\max}/\text{cm}^{-1}$ 3489 (NH), 3436 (NH), 3304 (br, 1° amide NH), 2839 (OCH_3), 1666 (C=O); δ_{H} (200 MHz, d_6 -DMSO) 3.88 (s, 3H, $-\text{OCH}_3$), 7.24-7.32 (m, 2H, Ar-4/6H), 7.41-7.49 (d, 1H, Ar-5H, $J = 7.4$), 7.49 (s, 1H, -NH), 7.59-7.73 (m, 4H, -NH, Ph-3'/4'/5'H) 8.04-8.08 (dd, 2H, Ph-2'/6'H, $J = 7.8$), 9.85 (s, 1H, -NH); m/z (EI) 270 (M^+ , 74.6%), 252 ($[\text{M}-\text{H}_2\text{O}]^+$), 239 ($[\text{M}-\text{OCH}_3]^+$), 105 ($\text{PhC}=\text{O}^+$), 77 (Ph^+).

50 3-Methyl-2-N-benzoylaminobenzamide

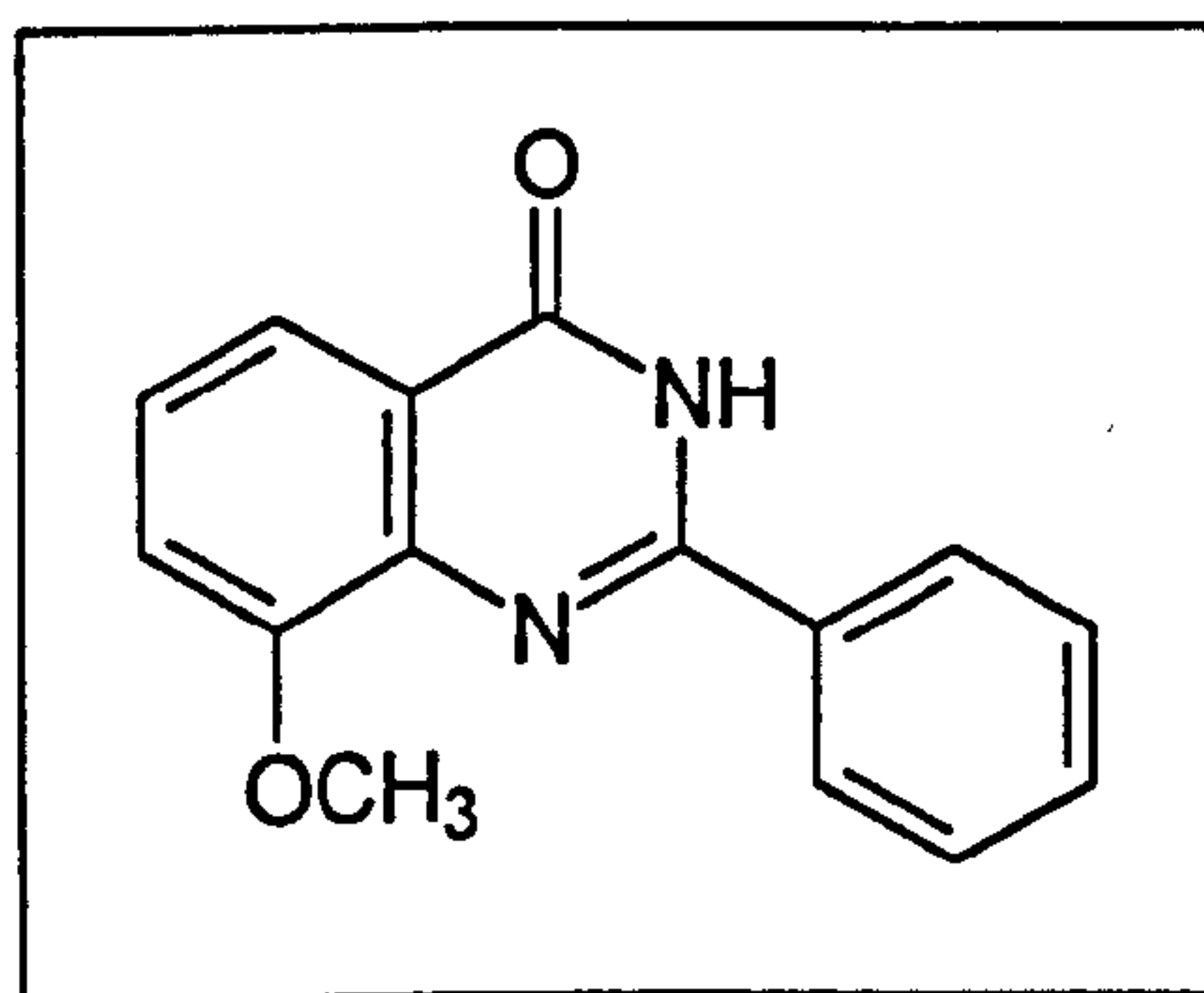


Prepared according to Standard Procedure A from 3-methyl-2-aminobenzamide **44** (0.5 g, 3.3 mmol) and benzoyl chloride (0.43 ml, 3.6 mmol). The product was recrystallised from methanol/water and recovered as white crystals (0.62 g, 2.5 mmol, 74%), m.p. 190-193 °C.

Anal. found C 70.19, H 5.39, N 10.53, $C_{15}H_{14}N_2O_2 \cdot 0.1$ mol.

H_2O requires C 70.35, H 5.59, N 10.94%; ν_{max}/cm^{-1} 3369 (NH), 3319 (NH), 3193 (br, 1° amide NH), 3023, 2963, 2923 (CH_3), 1657 (C=O); δ_H (200 MHz, d_6 -DMSO) 2.33 (s, 3H, - CH_3), 7.33-7.40 (t, 1H, Ar-5H, $J = 7.5$), 7.49-7.60 (m, 3H, -NH and Ar-4/6H), 7.63-7.71 (m, 3H, Ph-3'/4'/5'H), 7.84 (s, 1H, -NH), 8.04-8.09 (dd, 2H, Ph-2'/6'H, $J = 1.6, 7.9$), 10.34 (s, 1H, -NH); m/z (EI) 254 (M^+ , 23.5%), 237 ($[M-OH]^+$), 105 (PhC=O $^+$), 77 (Ph $^+$).

51 8-Methoxy-2-phenylquinazolin-4-[3H]-one



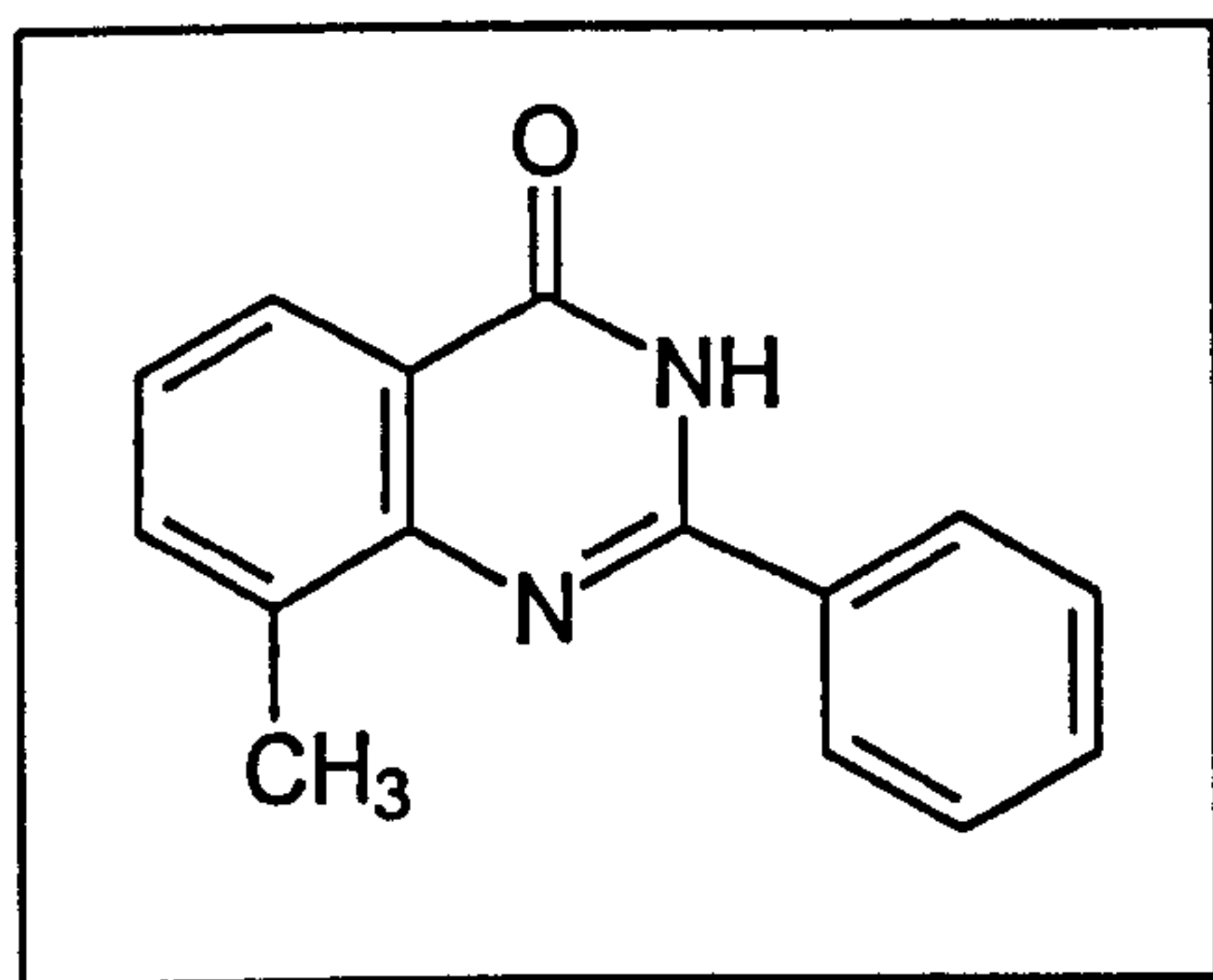
A. Prepared according to Standard Procedure B from 3-methoxy-2-N-benzoylaminobenzamide **49** (0.2 g, 0.74 mmol), in 2% aq. NaOH solution (2 ml) at room temperature (12 hours). The product was isolated as white crystals after recrystallisation from methanol/water (0.12 g, 0.48 mmol, 65%), m.p. 252-256 °C.

B. Prepared according to Standard Procedure C from 3-methoxy-2-aminobenzamide **41** (1.0 g, 6 mmol) and benzoyl chloride (0.8 ml, 6.6 mmol). The product was recrystallised from methanol/water to give white crystals (1.2 g, 4.5 mmol, 75%), m.p. 252-256 °C.

Anal. found C 71.38, H 4.39, N 11.17, $C_{15}H_{12}N_2O_2$ requires C 71.42, H 4.79, N 11.10%; ν_{max}/cm^{-1} 3191 and 3162 (2° amide NH), 2836 (OCH_3), 1662 (C=O); δ_H (200 MHz, d_6 -DMSO) 4.06 (s, 3H, - OCH_3), 7.47-7.61 (m, 2H, Ar-6/7H), 7.63-7.69 (m, 3H, Ph-3'/4'/5'H),

7.80-7.85 (dd, 1H, Ar-5H, $J = 1.8, 7.5$), 8.27-8.32 (m, 2H, Ph-2'/6'H) 12.70 (s, 1H, -NH); m/z (EI) 252 (M^+ , 100%), 251 ($[M-H]^+$), 223 ($[M-CHO]^+$), 77 (Ph^+).

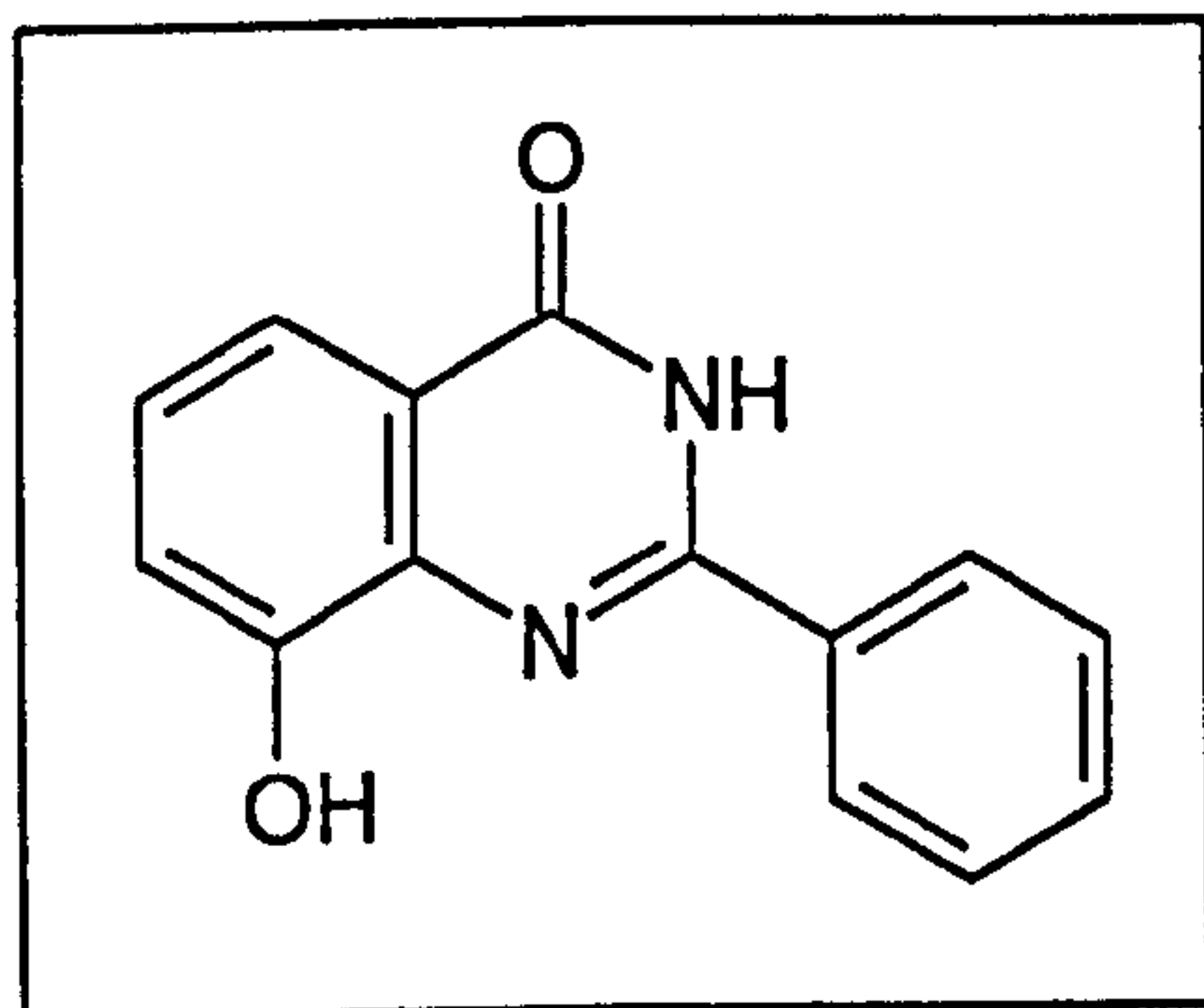
52 8-Methyl-2-phenylquinazolin-4-[3H]-one



Prepared according to Standard Procedure B from 3-methyl-2-*N*-benzoylaminobenzamide **50** (0.1 g, 0.4 mmol), in 2% aq. NaOH solution (25 ml) at room temperature (2 hours). The product was recrystallised from methanol/water to give white crystals (0.07 g, 0.3 mmol, 72%), m.p. 206-209 °C.

Anal. found C 75.39, H 4.99, N 11.81, $C_{15}H_{12}N_2O \cdot 0.1$ mol. H_2O requires C 75.67, H 5.17, N 11.71%; ν_{max}/cm^{-1} 3165 (2° amide NH), 2954 and 2922 (CH_3), 1675 (C=O); δ_H (200 MHz, d_6 -DMSO) 2.72 (s, 3H, $-CH_3$), 7.45-7.53 (t, 1H, Ar-6H, $J = 7.6$), 7.64-7.68 (dd, 3H, Ph-3'/4'/5'H, $J = 2.0, 5.2$), 7.77-7.81 (d, 1H, Ar-7H, $J = 7.1$), 8.07-8.10 (d, 1H, Ar-5H, $J = 7.1$), 8.30-8.35 (dd, 2H, Ph-2'/6'H, $J = 7.9$), 12.65 (s, 1H, -NH); m/z (EI) 236 (M^+ , 100%), 143 ($[M-CH_3Ph]^+$), 77 (Ph^+).

53 8-Hydroxy-2-phenylquinazolin-4-[3H]-one

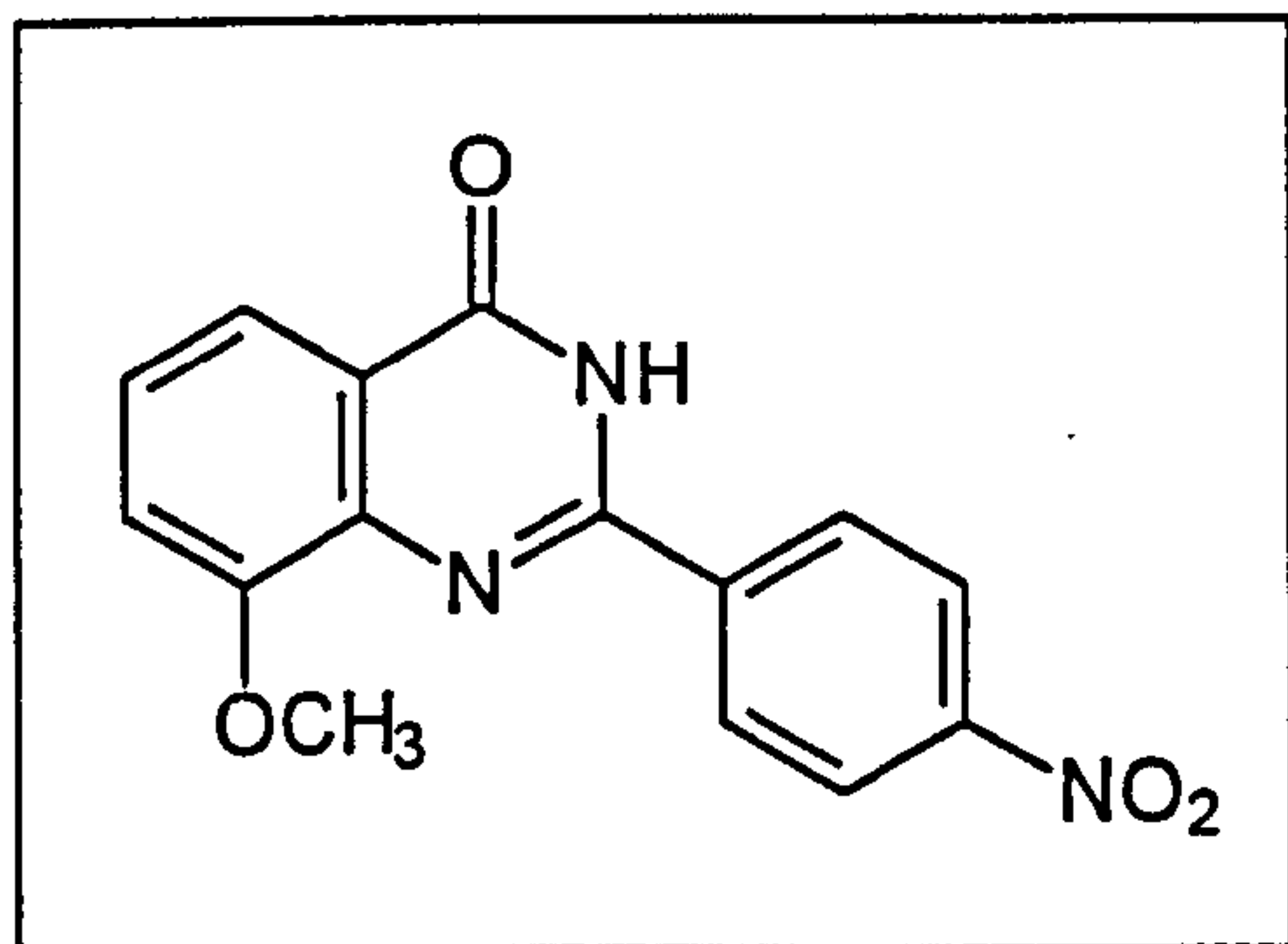


Prepared according to Standard Procedure D from 8-methoxy-2-phenylquinazolin-4-[3H]-one **51** (0.5 g, 2 mmol). The product (0.35 g) was recrystallised from propan-2-ol/water to give pale orange crystals (0.19 g, 0.75 mmol, 67%), m.p. 280-284 °C.

Anal. found C 69.54, H 4.05, N 11.46, $C_{14}H_{10}N_2O_2 \cdot 0.1$ mol. H_2O requires C 70.05, H 4.28, N 11.67%; ν_{max}/cm^{-1} 3380 (br, OH), 3152 and 3125 (2° amide NH), 1642 (C=O); δ_H (200 MHz, d_6 -DMSO) 7.33-7.37 (d, 1H, Ar-7H, $J = 7.8$), 7.42-7.50 (t, 1H, Ar-6H, $J = 7.8$), 7.66-7.72 (m, 4H, Ar-5H, Ph-3'/4'/5'H), 8.51-8.54 (dd, 2H, Ph-2'/6'H, $J =$

7.1), 9.75 (br s, 1H, -OH), 12.60 (br s, 1H, -NH) ; δ_C (d_6 -DMSO) 116.01, 118.68 (Ar-6/7C), 122.03 (C-Ph), 127.43-128.76 (Ph-1'/2'/3'/4'C), 131.50 (Ar-5C), 137.98 (Ar-8aC), 150.72 (Ar-8C), 153.31 (Ar-4aC), 162.62 (C=O); m/z (EI) 238 (M^+ , 100%), 77 (Ph^+).

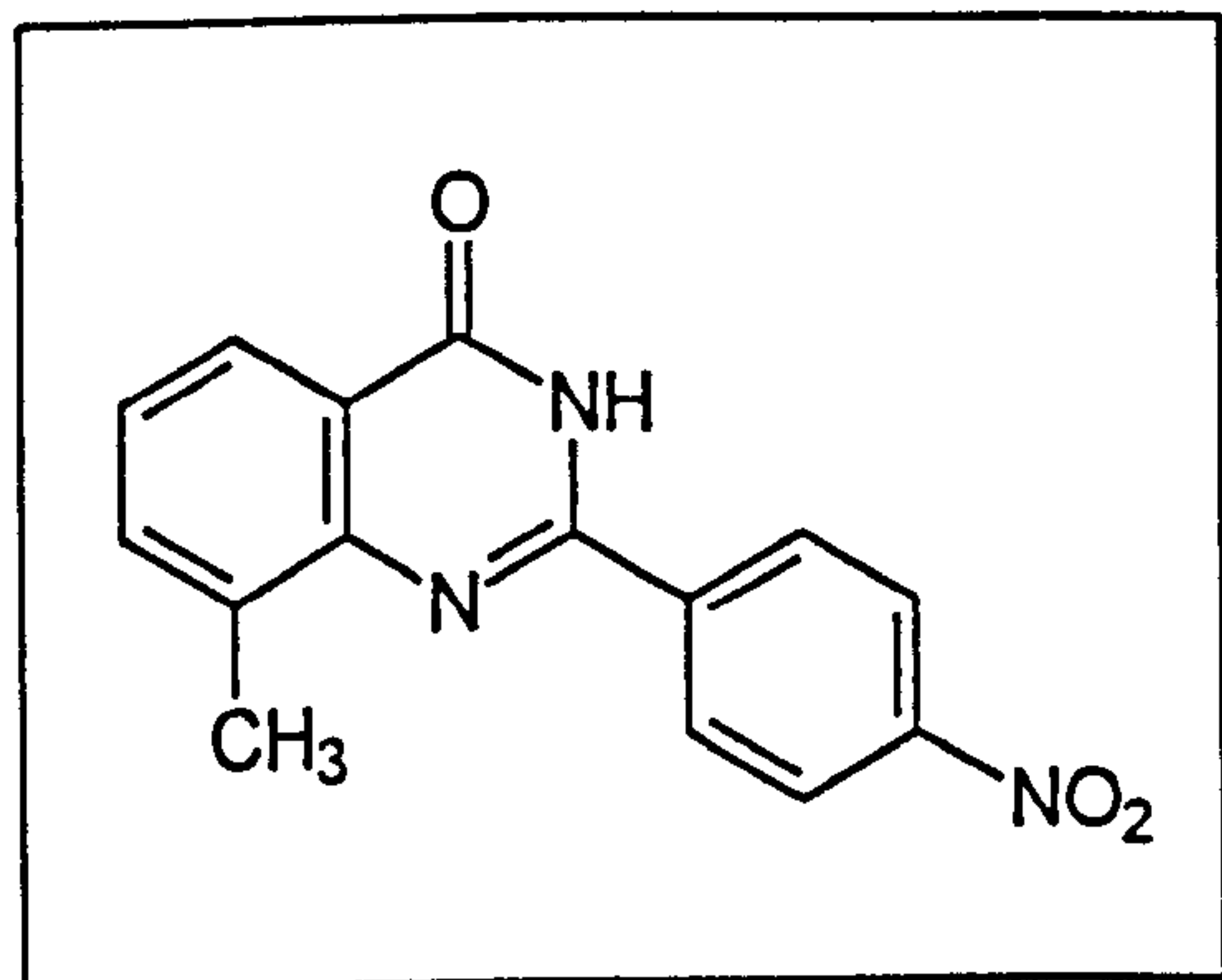
55 8-Methoxy-2-(4'-nitrophenyl)quinazolin-4-[3H]-one



Prepared according to **Standard Procedure B**, from 3-methoxy-2-*N*-(4'-nitrobenzoyl) aminobenzamide **54** (0.5 g, 1.6 mmol) in 10% aq. NaOH solution at 100 °C (2 hours). The product (0.1 g) was recrystallised from DMF/water at 100 °C to give yellow crystals (0.66 g, 0.2 mmol, 66%), m.p. 306-308 °C.

Anal. found C 59.16, H 3.92, N 13.99, $C_{13}H_{11}N_3O_4 \cdot 0.3$ mol. H_2O requires C 59.52, H 3.86, N 13.87%; ν_{max}/cm^{-1} 3037 (2° amide NH), 2837 (OCH_3), 1686 (C=O), 1522 (NO_2), 1348 (NO_2); δ_H (200 MHz, d_6 -DMSO) 4.06 (s, 3H, $-OCH_3$), 7.52-7.56 (d, 1H, Ar-7H, $J = 8.0$), 7.58-7.66 (t, 1H, Ar-6H, $J = 7.9$), 7.82-7.86 (d, 1H, Ar-5H, $J = 1.5, 7.7$), 8.50 (s, 4H, Ph-2'/3'/5'/6'H), 12.95 (br s, 1H, -NH); m/z (EI) 297 (M^+ , 100%), 279 ($[M-H_2O]^+$), 267 ($[M-OCH_2]^+$), 250 ($[M-HNO_2]^+$).

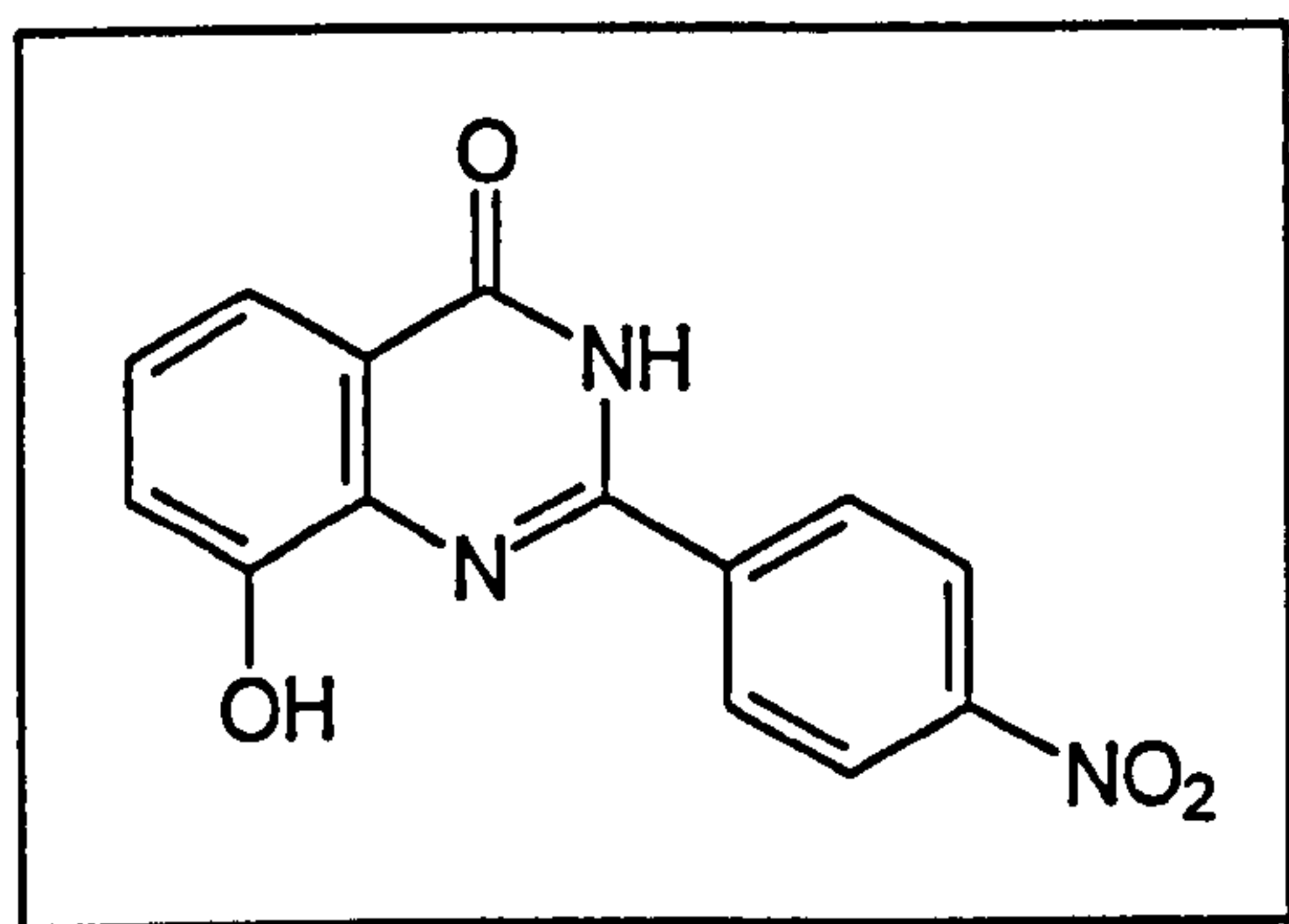
57 8-Methyl-2-(4'-nitrophenyl)quinazolin-4-[3H]-one



Prepared according to **Standard Procedure C** from 3-methyl-2-aminobenzamide **44** (0.5 g, 3.3 mmol) and 4-nitrobenzoyl chloride (0.93 g, 5.0 mmol). The product was isolated as a yellow crystalline solid from a saturated solution of aq. DMF, after stirring at 100 °C and carrying out a hot filtration (0.8 g, 2.9 mmol, 86%), m.p. 317-319 °C.

Anal. found C 64.54, H 3.91, N 14.91, $C_{15}H_{11}N_3O_3$ requires C 64.05, H 3.94, N 14.94%; $\nu_{\max}/\text{cm}^{-1}$ 3088 (2° amide NH), 2924 and 2904 (CH_3), 1683 ($\text{C}=\text{O}$), 1522 (NO_2), 1350 (NO_2); δ_{H} (200 MHz, d_6 -DMSO) 2.77 (s, 3H, $-\text{CH}_3$), 7.54-7.61 (t, 1H, Ar-6H, $J = 7.6$), 7.84-7.88 (d, 1H, Ar-7H, $J = 7.4$), 8.12-8.16 (d, 1H, Ar-5H, $J = 7.9$), 8.49-8.53 (d, 2H, Ph-2'/6'H, $J = 9.2$), 8.57-8.61 (d, 2H, Ph-3'/5'H, $J = 9.2$), 12.9 (s, 1H, $-\text{NH}$); m/z (EI) 281 (M^+ , 100%), 235 ($[\text{M}-\text{NO}_2]^+$), 77 (Ph^+).

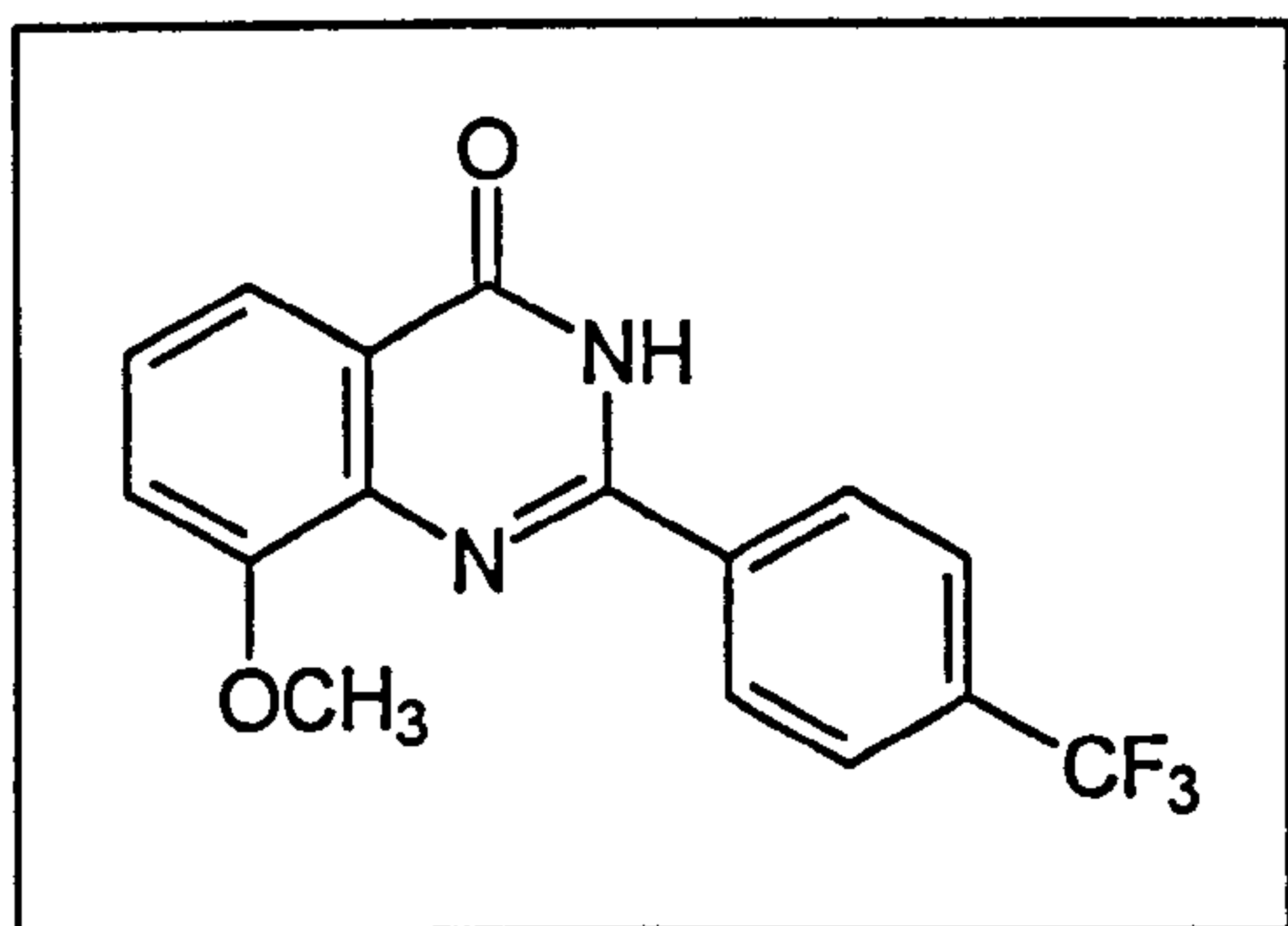
38 8-Hydroxy-2-(4'-nitrophenyl)quinazolin-4-[3H]-one



Prepared according to Standard Procedure D from 8-methoxy-2-(4'-nitrophenyl)quinazolin-4-[3H]-one 55 (0.2 g, 0.7 mmol). The product was isolated as a yellow crystalline solid after recrystallisation from propan-2-ol/water (0.07 g, 0.25 mmol, 38%), m.p. 321-323 °C.

Anal. found C 56.79, H 2.74, N 13.58, $C_{14}H_9N_3O_4$ requires C 59.36, H 3.20, N 14.84%; $\nu_{\max}/\text{cm}^{-1}$ 3408 (OH), 3387 (2° amide NH), 1686 ($\text{C}=\text{O}$), 1523 (NO_2); δ_{H} (200 MHz, d_6 -DMSO) 7.35-7.39 (d, 1H, Ar-7H, $J = 7.7$), 7.47-7.54 (t, 1H, Ar-6H, $J = 7.8$), 7.69-7.73 (d, 1H, Ar-5H, $J = 7.6$), 8.45-8.49 (d, 2H, Ph-2'/6'H, $J = 8.8$), 8.75-8.79 (d, 2H, Ph-3'/5'H, $J = 8.8$), 9.98 (s, 1H, $-\text{OH}$), 12.85 (s, 1H, $-\text{NH}$); m/z (EI) 283 (M^+ , 100%), 237 ($[\text{M}-\text{NO}_2]^+$), 143 ($[\text{M}-\text{OHPh}-\text{NO}_2]^+$).

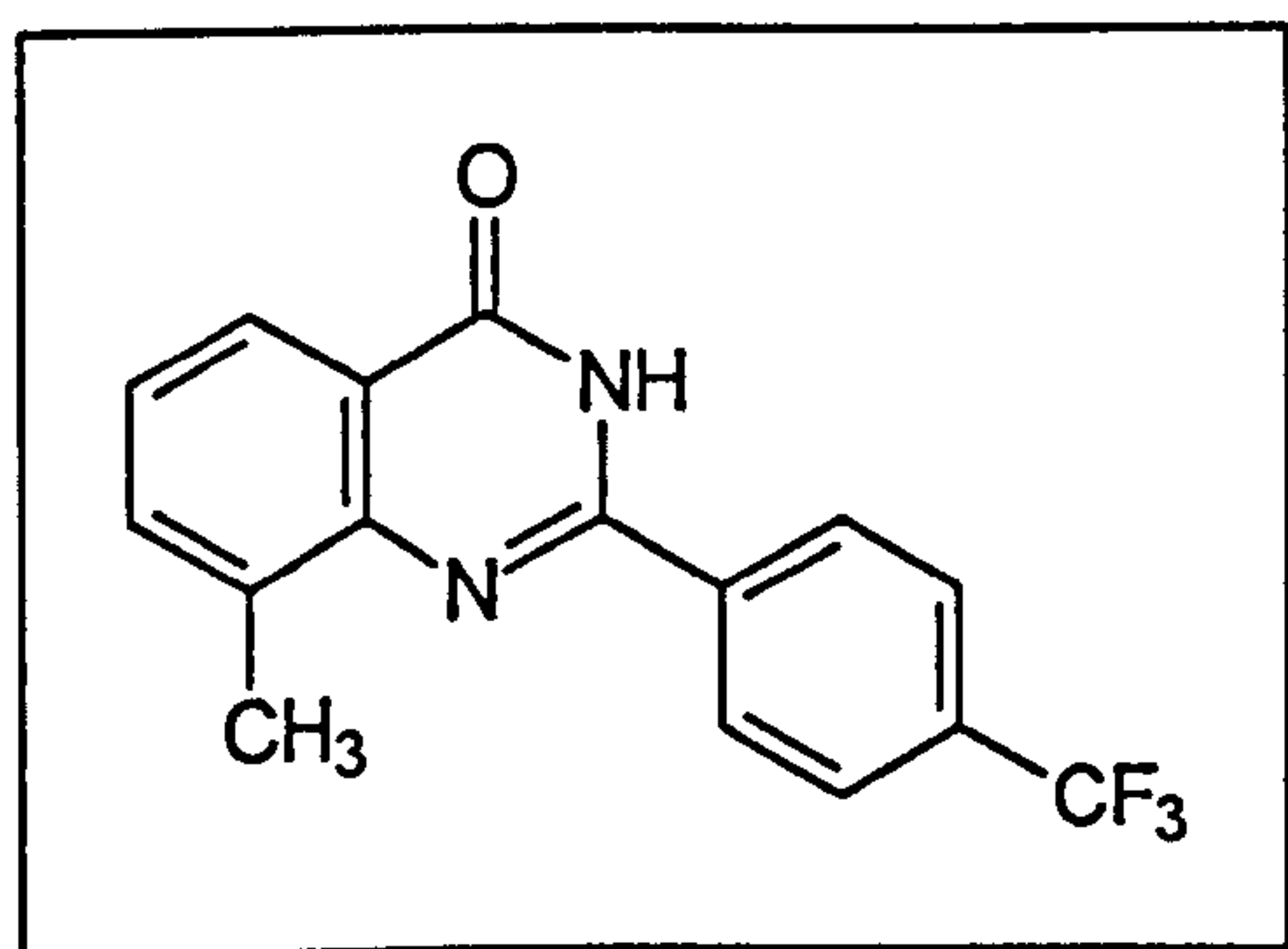
59 8-Methoxy-2-(4'-trifluoromethylphenyl)quinazolin-4-[3H]-one



Prepared according to Standard Procedure C from 3-methoxy-2-aminobenzamide 41 (0.2 g, 1.2 mmol) and 4-trifluoromethylbenzoyl chloride (0.2 ml, 1.3 mmol). The product was recrystallised from methanol/water to give white crystals (0.25 g, 0.8 mmol, 64%), m.p. 287-289 °C.

Anal. found C 59.89, H 3.37, N 8.69, $C_{16}H_{11}N_2O_2F_3$ requires C 60.00, H 3.46, N 8.75%; $\nu_{\max}/\text{cm}^{-1}$ 3116 (2° amide NH), 2843 (OCH_3), 1660 ($\text{C}=\text{O}$); δ_{H} (200 MHz, d_6 -DMSO) 4.06 (s, 3H, $-\text{OCH}_3$), 7.50-7.64 (m, 2H, Ar-6/7H), 7.81-7.84 (d, 1H, Ar-5H, $J = 7.0$), 8.02-8.06 (d, 2H, Ph-2'/6'H, $J = 8.4$), 8.45-8.49 (d, 2H, Ph-3'/5'H, $J = 8.4$), 12.91 (s, 1H, $-\text{NH}$); m/z (EI) 320 (M^+ , 100%), 310 ($[M-F]^+$), 291 ($[M-\text{OCH}_2]^+$), 145 ($\text{C}_6\text{H}_4\text{CF}_3^+$).

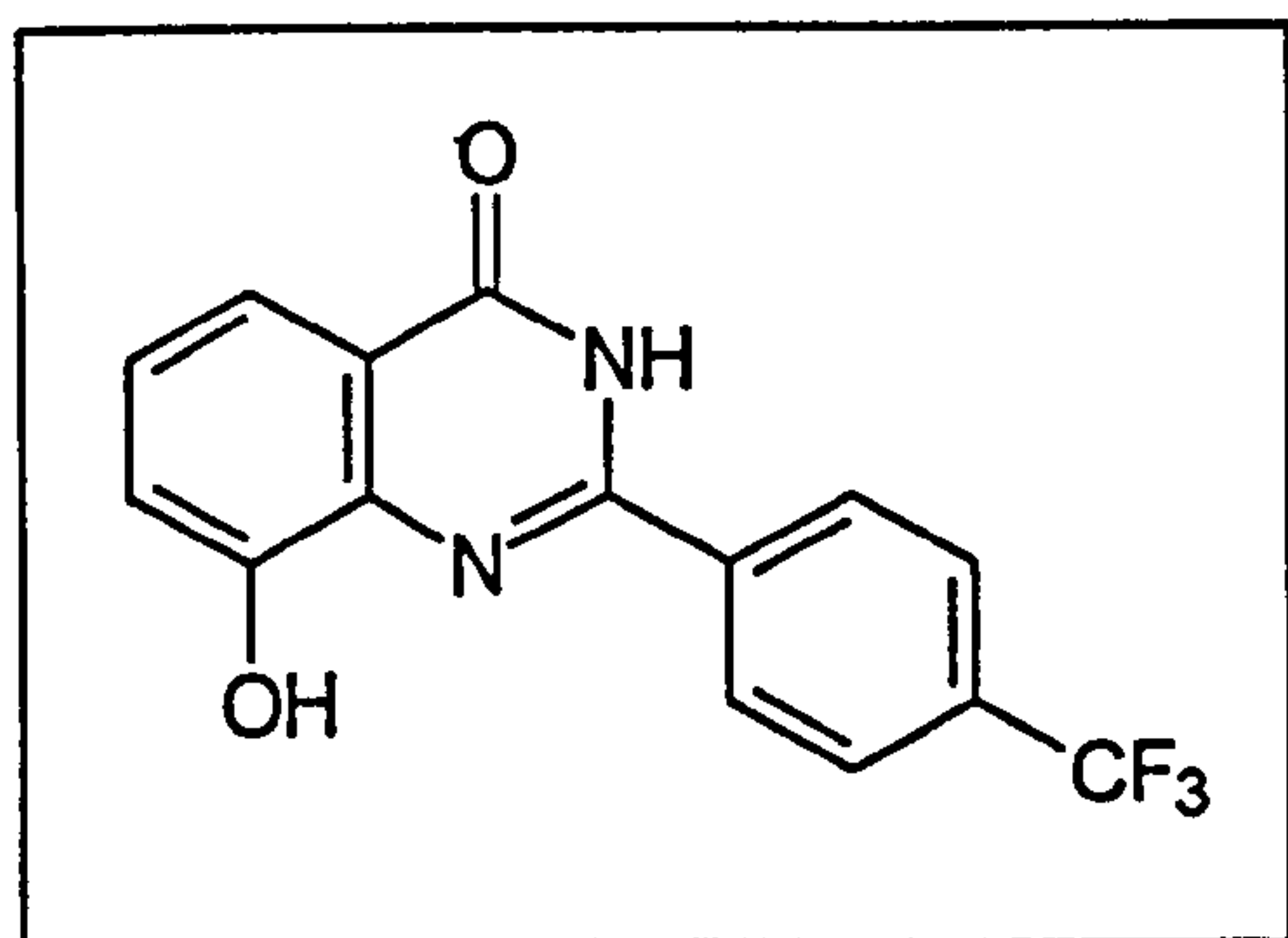
61 8-Methyl-2-(4'-trifluoromethylphenyl)quinazolin-4-[3H]-one



Prepared according to Standard Procedure C from 3-methyl-2-aminobenzamide 44 (0.2 g, 1.3 mmol) and 4-trifluoromethylbenzoyl chloride (0.22 ml, 1.5 mmol). The product was obtained after recrystallisation from methanol/water as white crystals (0.26 g, 0.9 mmol, 65%), m.p. 255-257 °C.

Anal found C 63.06, H 3.33, N 9.21, $C_{16}H_{11}N_2\text{OF}_3$ requires C 63.15, H 3.64, N 9.21%; $\nu_{\max}/\text{cm}^{-1}$ 3175 (2° amide NH), 1666 ($\text{C}=\text{O}$), 1336 (C-F), 1320 (C-F); δ_{H} (200 MHz, d_6 -DMSO) 2.75 (s, 3H, $-\text{CH}_3$), 7.56 (t, 1H, Ar-6H), 7.83 (d, 1H, Ar-7H), 8.08 (m, 3H, Ar-5H, Ph-3'/5'H), 8.51 (d, 2H, Ph-2'/6'H), 12.85 (s, 1H, $-\text{NH}$); m/z (EI) 304 (M^+ , 100%).

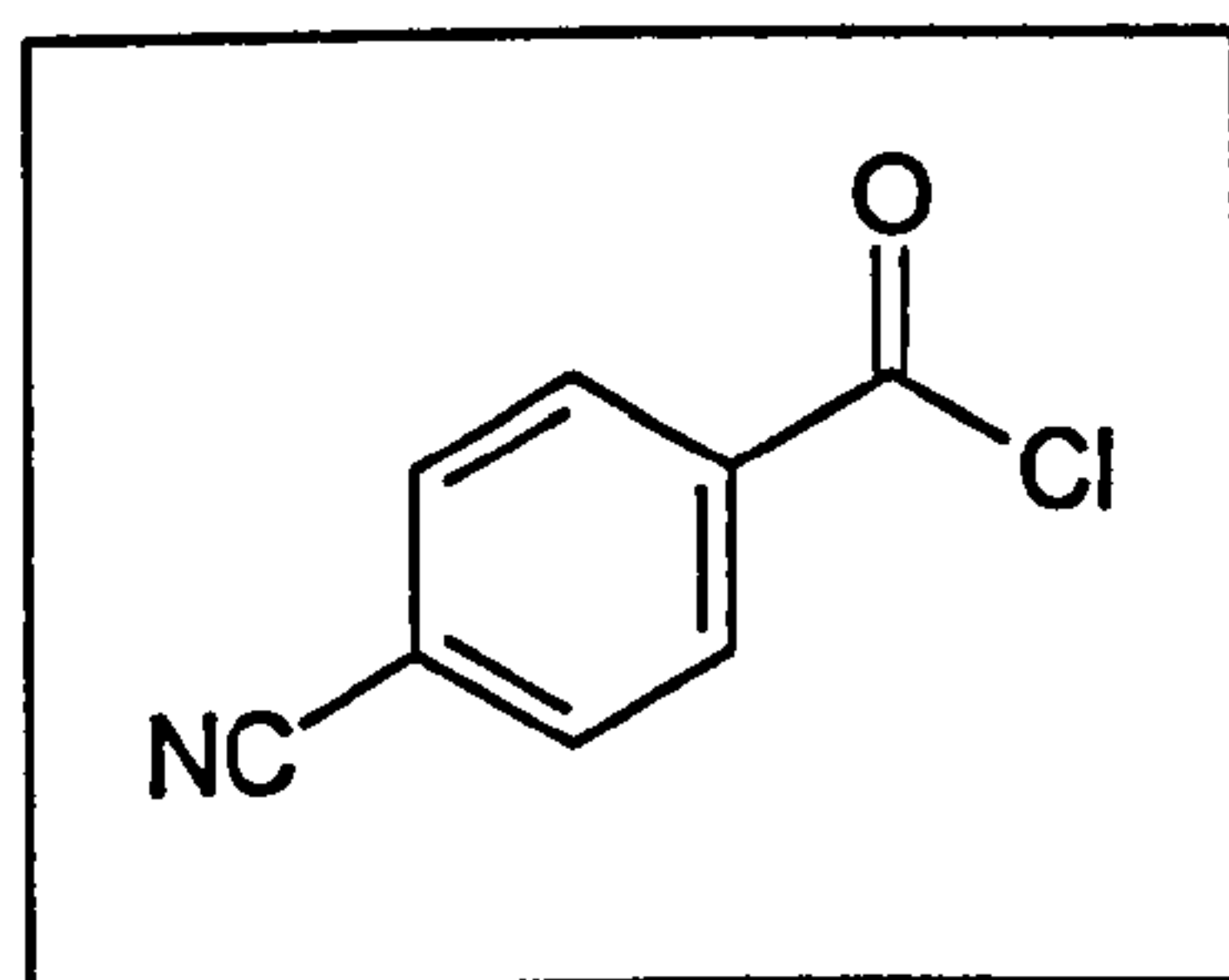
62 8-Hydroxy-2-(4'-trifluoromethylphenyl)quinazolin-4-[3H]-one



Prepared according to Standard Procedure D from 8-methoxy-2-(4'-trifluoromethylphenyl)quinazolin-4-[3H]-one **59** (0.1 g, 0.3 mmol). The product (51.8 mg) was purified by column chromatography, using EtOAc:petrol 40/60 (4:6) to give the product as a pale orange solid (17.7 mg, 0.06 mmol, 19%).

Anal. found[†] C 54.41, H 3.15, N 7.91, $C_{15}H_9N_2O_2F_3 \cdot 1.5$ mol. HCl requires C 54.06, H 3.63, N 8.41%; δ_H (200 MHz, d_6 -DMSO) 7.32-7.37 (dd, 1H, Ar-7H, $J = 1.6, 7.9$), 7.44-7.52 (t, 1H, Ar-6H, $J = 7.8$), 7.67-7.71 (dd, 1H, Ar-5H, $J = 1.5, 7.8$), 8.00-8.05 (d, 2H, Ph-3'/5'H, $J = 8.4$), 8.69-8.73 (d, 2H, Ph-2'/6'H, $J = 8.1$), 9.95 (br s, 1H, -OH), 12.8 (br s, 1H, -NH); m/z (EI) 306 (M^+ , 100%), 172 ($[M-PhF_3]^+$).

63 4-Cyanobenzoyl chloride



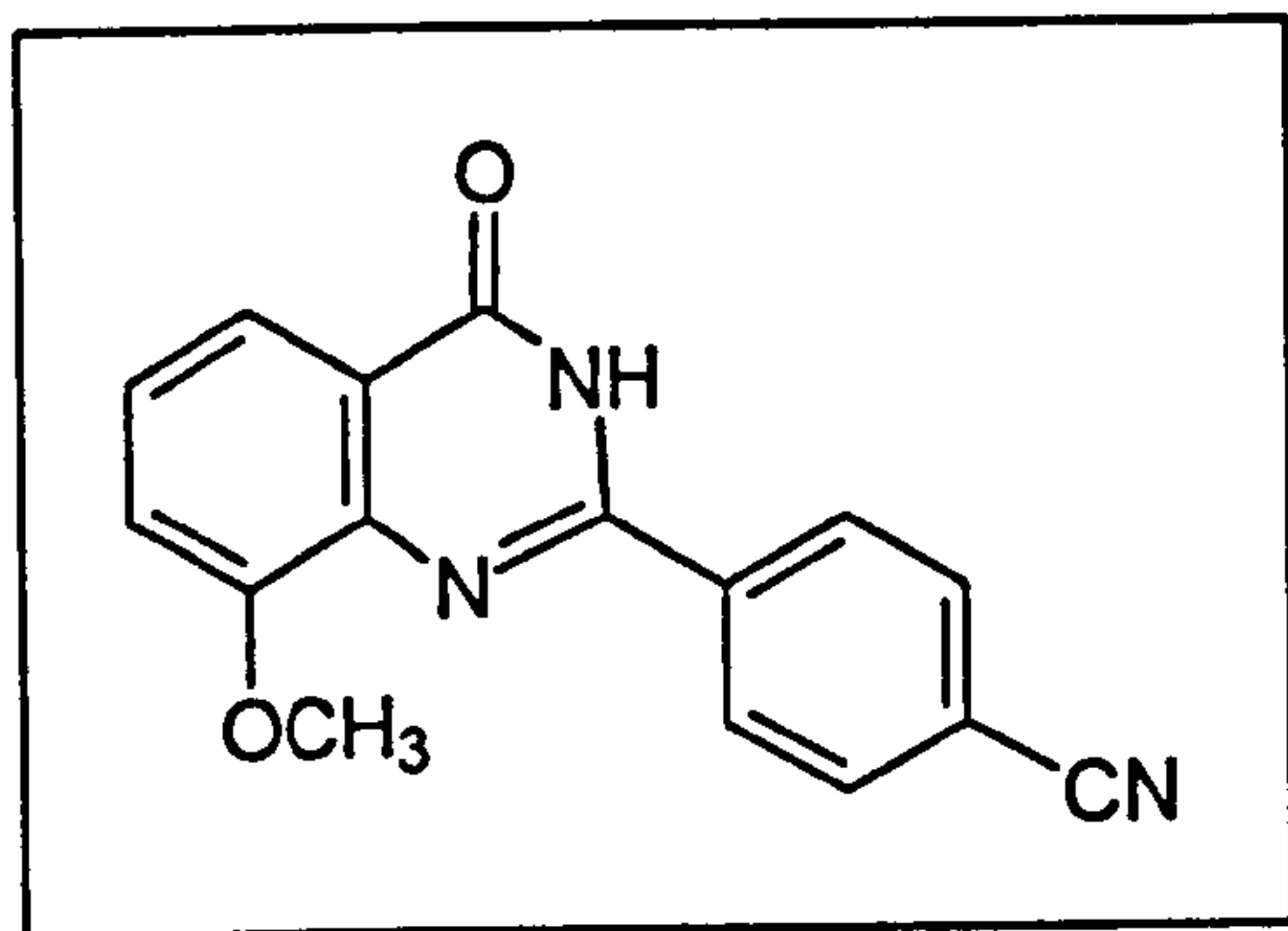
4-Cyanobenzoic acid (1.0 g, 6.8 mmol) was suspended in thionyl chloride (5 ml) and the mixture was boiled under reflux for 2 hours. The reaction mixture was cooled and the solvent was removed under water pressure to give a cream solid (1.04 g, 6.3 mmol, 92%), m.p. 67-68 °C (64-65 °C)²⁵¹

A small amount of product was converted to the corresponding methyl ester, for NMR analysis: 10 mg was dissolved in dry THF (5 ml) and stirred at room temperature. Methanol (3 ml) was added and the solution was stirred for 30 minutes. The solvent was removed under vacuum to leave a cream solid.

δ_H (200 MHz, d_6 -DMSO – methyl 4-cyanobenzoate) 4.01 (s, 3H, -OCH₃), 8.10-8.14 (dd, 2H, Ar-2/6H, $J = 8.4$), 8.19-8.23 (dd, 2H, Ar-3/5H, $J = 8.0$)

[†] Figures given are not within 0.4% of theoretical values.

65 8-Methoxy-2-(4'-cyanophenyl)quinazolin-4-[3H]-one

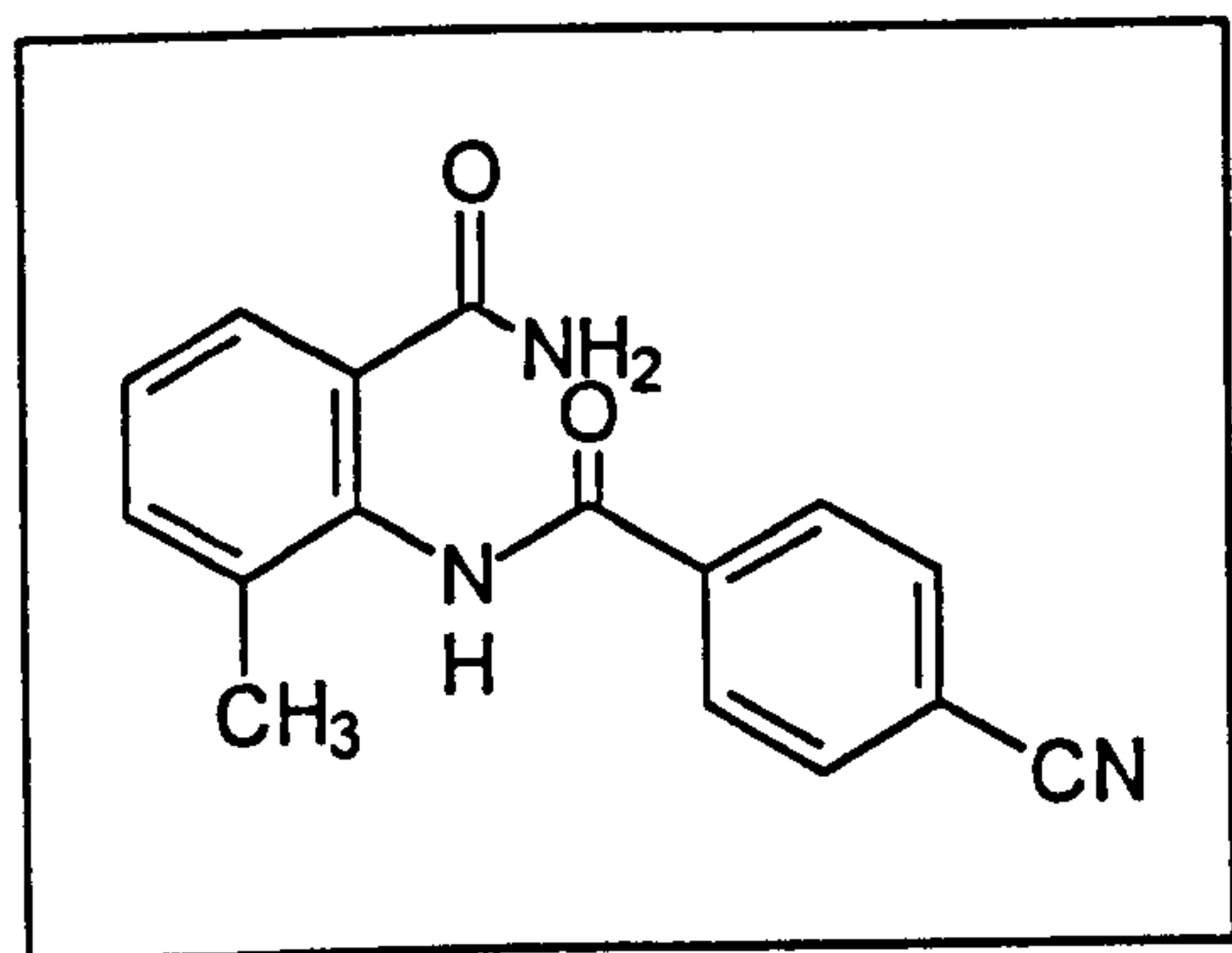


Prepared according to Standard Procedure C from 3-methoxy-2-aminobenzamide **41** (0.5 g, 3.0 mmol) and 4-cyanobenzoyl chloride **63** (0.55 g, 3.3 mmol). The product (0.68 g) was purified by column chromatography, using DCM:MeOH (92:8) as the eluent, to give a cream solid (0.28 g, 0.82 mmol, 27%),

m.p. 306-309 °C.

Anal. found C 66.19, H 4.16, N 14.13, $C_{16}H_{11}N_3O_2 \cdot 0.75 \text{ mol. H}_2\text{O}$ requires C 66.08, H 4.33, N 14.45%; $\nu_{\text{max}}/\text{cm}^{-1}$ 3413 (br, 2° amide NH), 2841 (OCH_3), 2230 (CN), 1681 (CO); δ_{H} (200 MHz, d_6 -DMSO) 4.05 (s, 3H, $-\text{OCH}_3$), 7.48-7.53 (dd, 1H, Ar-7H, $J = 1.5, 8.1$), 7.55-7.63 (t, 1H, Ar-6H, $J = 7.8$), 7.79-7.83 (dd, 1H, Ar-5H, $J = 1.5, 7.7$), 8.12-8.15 (d, 2H, Ph-2'/6'H, $J = 8.5$), 8.41-8.45 (d, 2H, Ph-3'/5'H, $J = 8.5$), 12.95 (s, 1H, $-\text{NH}$); m/z (EI) 277 (M^+ , 63%), 248 ($[\text{M}-\text{OCH}_3]^+$), 78 (PhH^+).

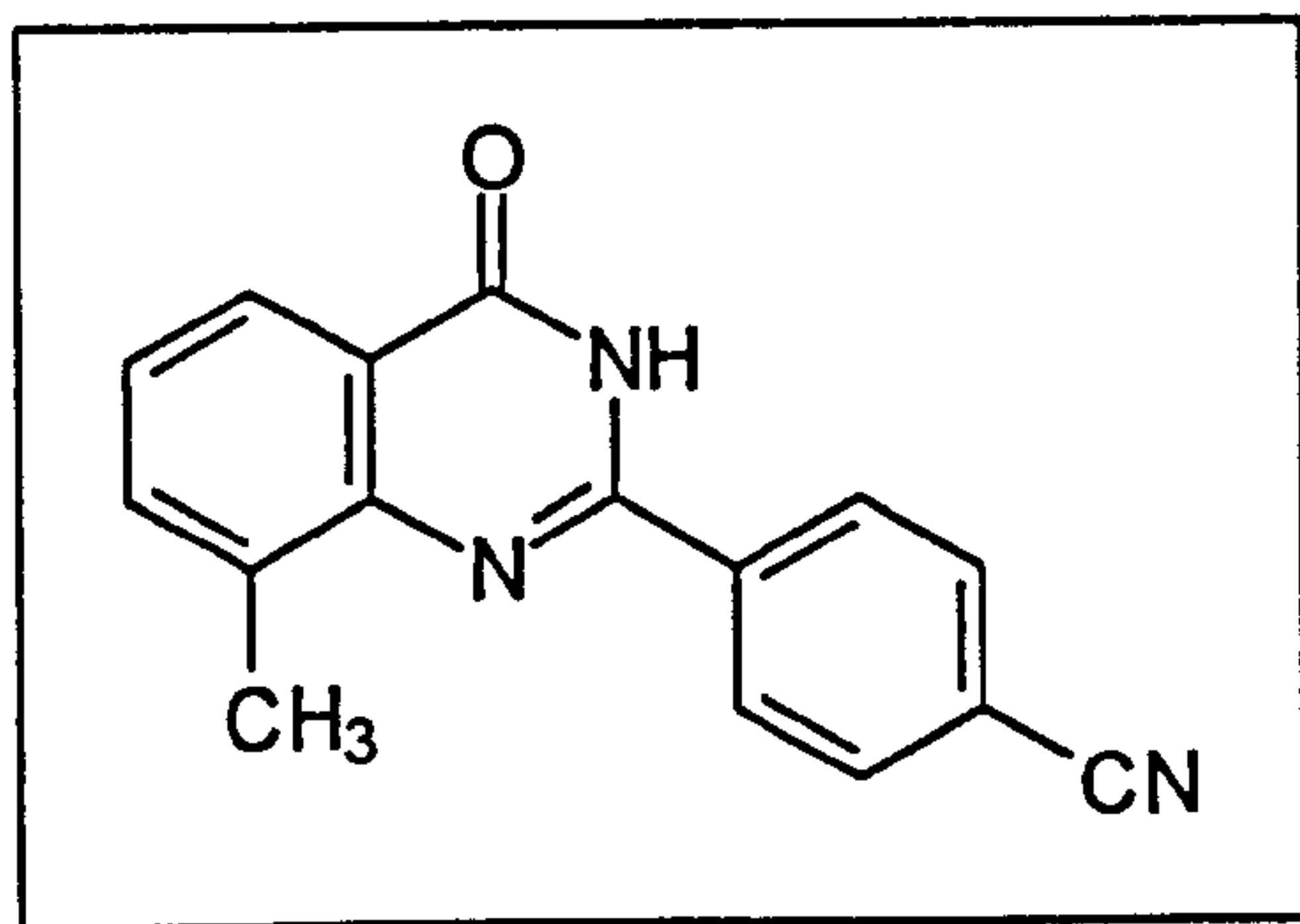
66 3-Methyl-2-N-(4'-cyanobenzoyl) aminobenzamide



Prepared according to Standard Procedure A from 3-methyl-2-aminobenzamide **44** (1.0 g, 6.7 mmol) and 4-cyanobenzoyl chloride **63** (1.7 g, 10.0 mmol). The product was purified by column chromatography using DCM:MeOH (97:3) as eluent to give a white solid (0.62 g, 2.4 mmol, 36%), m.p. 299-301 °C.

Anal. found C 68.51, H 4.35, N 14.66, $C_{16}H_{13}N_3O_3$ requires C 68.80, H 4.69, N 15.05%; $\nu_{\text{max}}/\text{cm}^{-1}$ 3431 (br, NH), 3191 (NH), 2969 (CH_3), 2226 (CN), 1666 (C=O); δ_{H} (200 MHz, d_6 -DMSO) 2.31 (s, 3H, CH_3), 7.33-7.37 (d, 1H, Ar-7H, $J = 7.6$), 7.41-7.53 (m, 3H, Ar-5/6H, $-\text{NH}$), 7.83 (s, 1H, $-\text{NH}$), 8.07-8.14 (d, 2H, Ph-2'/6'H, $J = 8.5$), 8.18-8.22 (d, 2H, Ph-3'/5'H, $J = 8.5$); m/z (EI) 279 (M^+ , 6%), 234 ($[\text{M}-\text{CONH}_2]^+$).

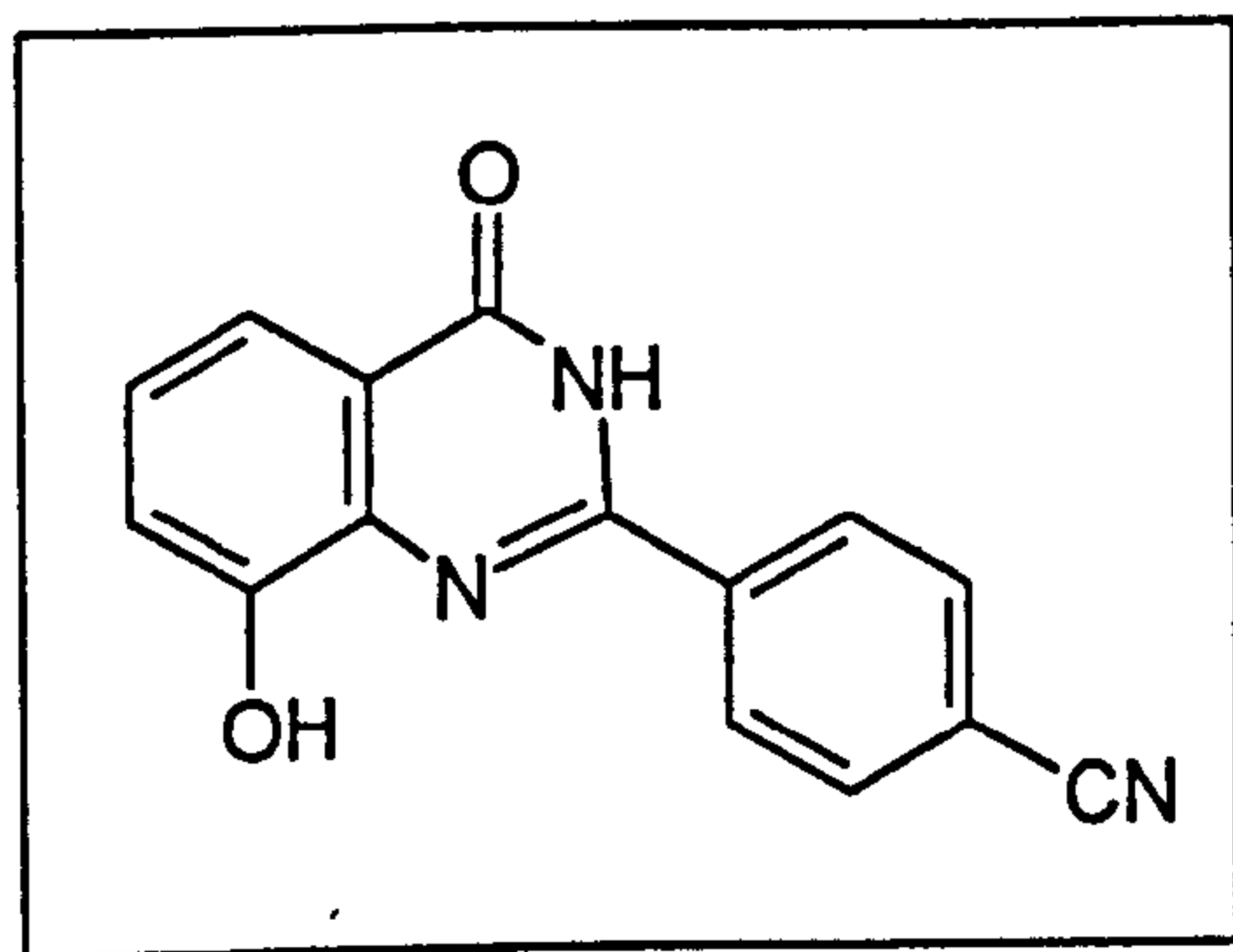
67 8-Methyl-2-(4'-cyanophenyl)quinazolin-4-[3H]-one



Prepared according to Standard Procedure C from 3-methyl-2-aminobenzamide 44 (0.39 g, 2.6 mmol) and 4-cyanobenzoyl chloride 63 (0.48 g, 2.9 mmol). The product was purified by column chromatography, using DCM:MeOH (92:8) as eluent to give a white solid (0.15 g, 0.56 mmol, 21%), m.p. 330-332 °C.

Anal. found C 73.27, H 4.16, N 15.89, $C_{16}H_{11}N_3O$ requires C 73.55, H 4.24, N 16.09%; $\nu_{\max}/\text{cm}^{-1}$ 3170 (2° amide NH), 2228 (CN), 1683 (C=O); δ_H (200 MHz, d_6 -DMSO) 2.73 (s, 3H, -CH₃), 7.51-7.58 (t, 1H, Ar-6H, $J = 7.6$), 7.81-7.85 (d, 1H, Ar-7H, $J = 6.6$), 8.09-8.17 (m, 3H, Ar-5H and Ph-2'/6'H, $J = 8.7$), 8.47-8.51 (d, 2H, Ph-3'/5'H, $J = 8.7$), 12.9 (s, 1H, -NH); m/z (EI) 261 (M^+ , 100%).

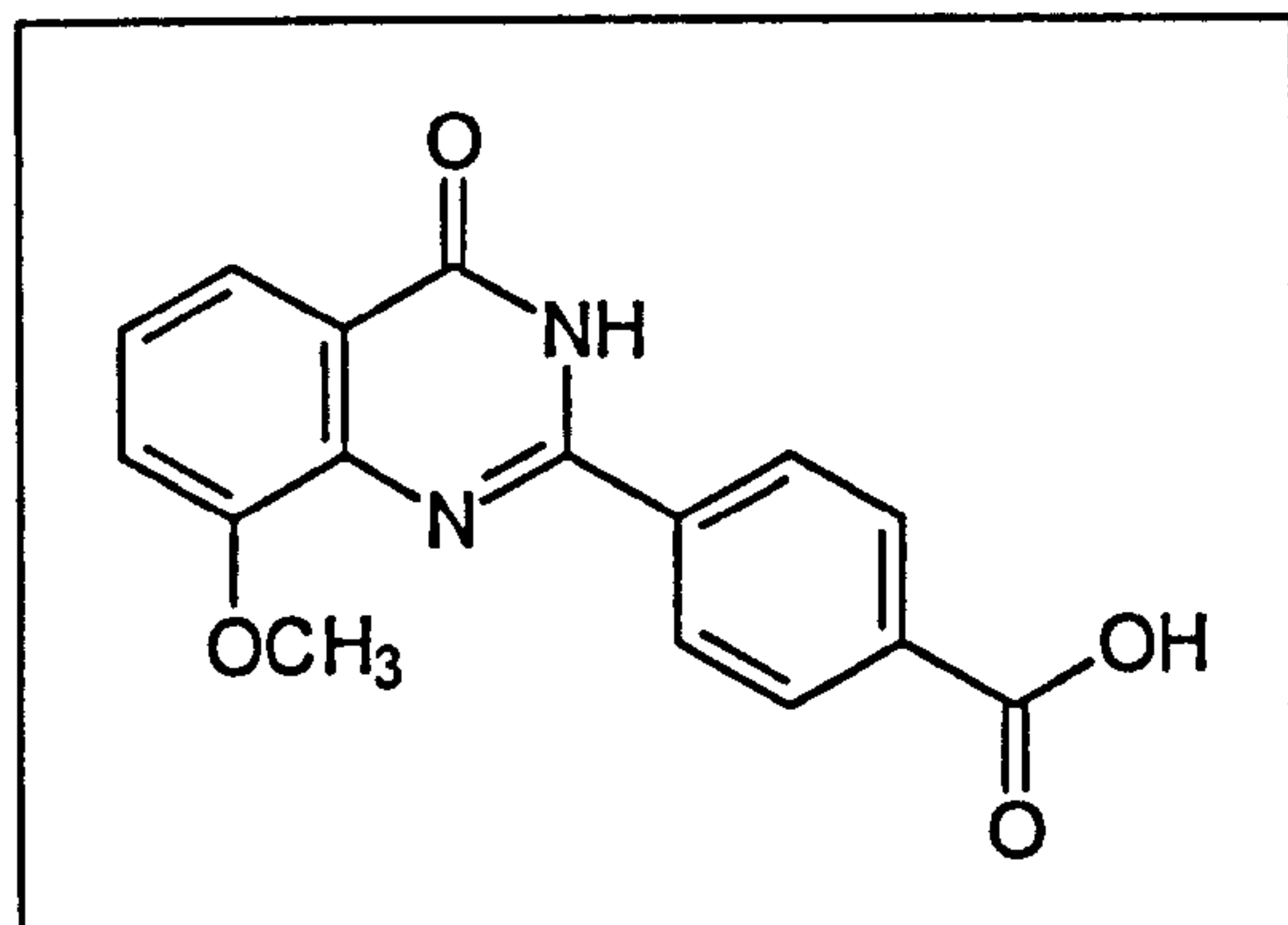
68 8-Hydroxy-2-(4'-cyanophenyl)quinazolin-4-[3H]-one



Prepared according to Standard Procedure D from 8-methoxy-2-(4'-cyanophenyl)quinazolin-4-[3H]-one 65 (0.2 g, 0.72 mmol). The product (108 mg) was purified by column chromatography, using DCM:MeOH (95:5) as the eluent, to give a cream solid (26.4 mg, 0.1 mmol, 14%).

Anal. found[‡] C 67.03, H 3.89, N 13.77, $C_{15}H_9N_3O_2 \cdot 0.5 \text{ mol. CH}_3\text{OH}$ requires C 66.66, H 3.97, N 15.05%; δ_H (200 MHz, d_6 -DMSO) 7.39-7.43 (dd, 1H, Ar-7H, $J = 1.4, 7.9$), 7.51-7.59 (t, 1H, Ar-6H, $J = 7.9$), 7.73-7.77 (dd, 1H, Ar-5H, $J = 1.4, 7.8$), 8.19-8.23 (d, 2H, Ph-2'/6'H, $J = 8.5$), 8.75-8.79 (d, 2H, Ph-3'/5'H, $J = 8.5$), 9.9 (s, 1H, -NH); m/z (EI) 263 (M^+ , 100%), 143 ($[M-\text{PhCNOH}]^+$).

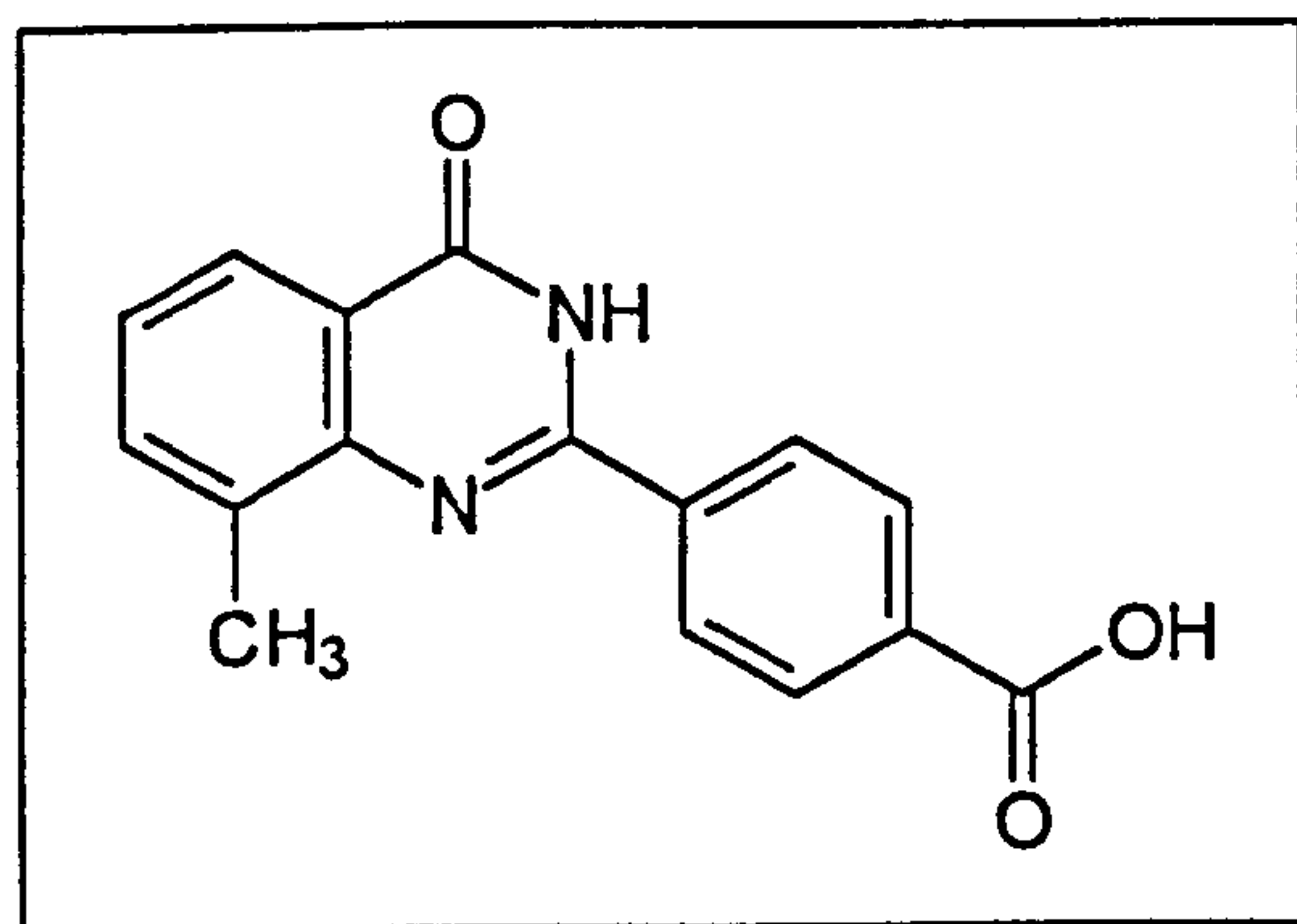
[‡] Figures given are not within 0.4% of theoretical values.

71 8-Methoxy-2-(4'-carboxyphenyl)quinazolin-4-[3H]-one²⁶⁸

8-Methoxy-2-(4'-cyanophenyl)quinazolin-4-[3H]-one **65** (0.5 g, 1.8 mmol) was suspended in ethanol (50 ml). 5 M (20%) aq. NaOH solution (20 ml) was added and the reaction mixture was refluxed for 4 hours. The ethanol was removed under reduced pressure and the pH of the remaining aq. solution

was adjusted to 6.5, whereupon a pale yellow precipitate formed. The precipitate was collected by filtration and cream/yellow product was washed with water and a small amount of methanol (0.68 g, 2.3 mmol, >100% crude).

δ_{H} (200 MHz, d_6 -DMSO) 4.05 (s, 3H, -OCH₃), 7.50-7.52 (d, 1H, Ar-7H, $J = 6.7$), 7.56-7.61 (t, 1H, Ar-6H, $J = 7.9$), 7.80-7.82 (d, 1H, Ar-5H, $J = 6.4$), 8.16-8.19 (d, 2H, Ph-2'/6'H, $J = 8.4$), 8.36-8.39 (d, 2H, Ph 3'/5'H, $J = 8.4$).

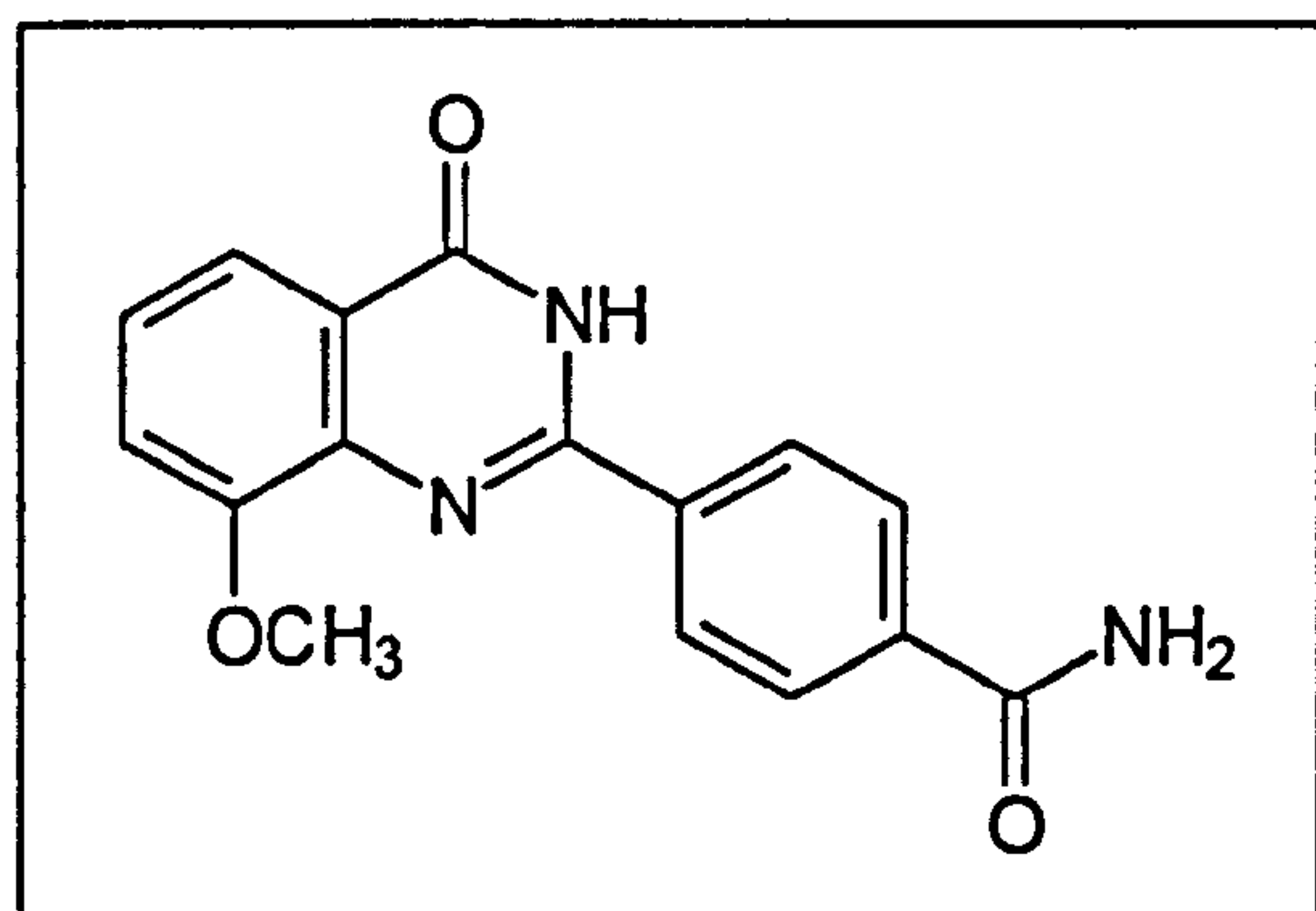
72 8-Methyl-2-(4'-carboxyphenyl)quinazolin-4-[3H]-one²⁶⁸

Prepared in a similar way to compound **71** from 8-methyl-2-(4'-cyanophenyl)quinazolin-4-[3H]-one **67** (0.1 g, 0.4 mmol) to give the product as a cream solid.

Crude yield : 0.98 g, 0.4 mmol, 92%.

$\nu_{\text{max}}/\text{cm}^{-1}$ 3166 (2° amide NH), 3049 (OH), 1696

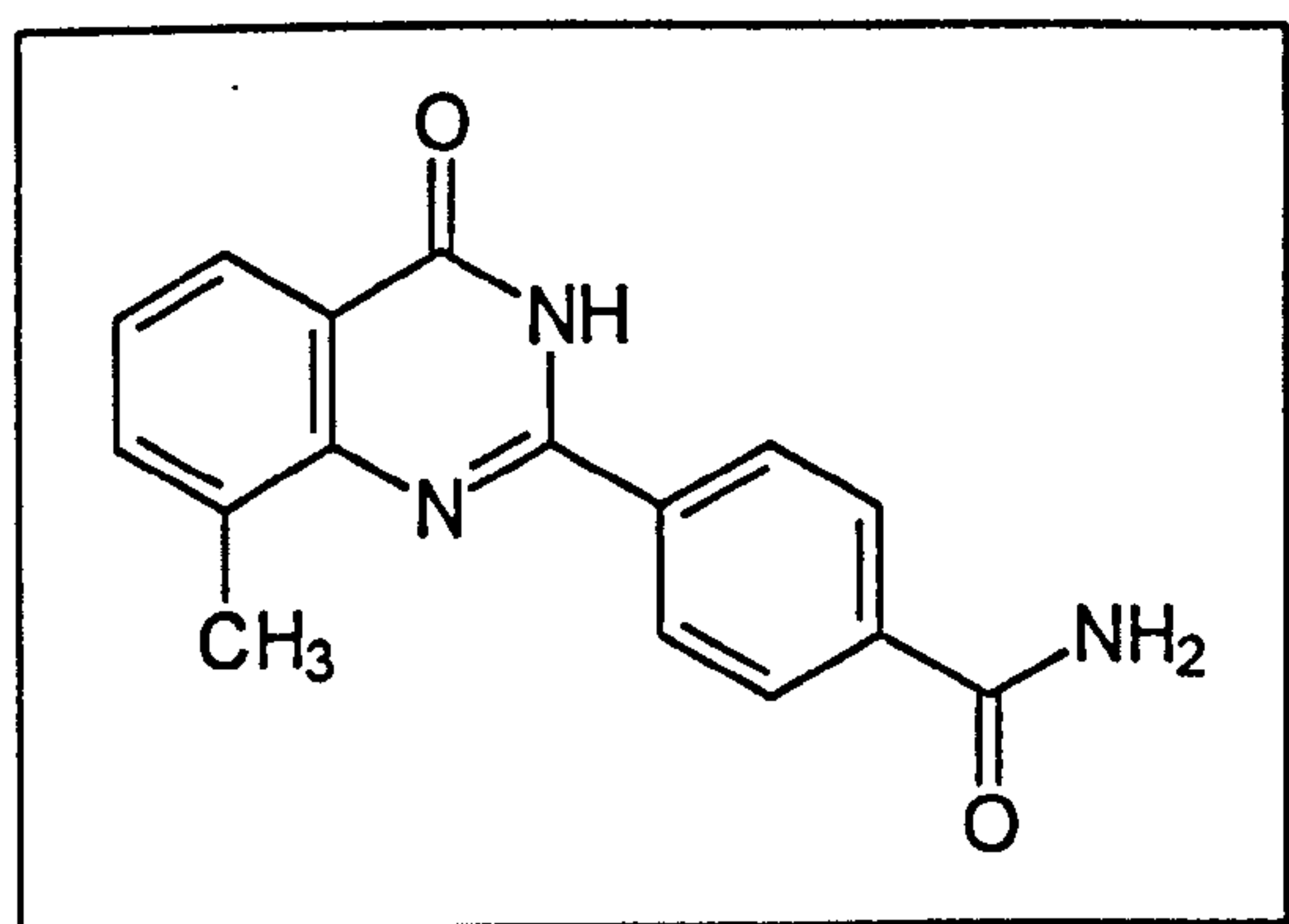
(CO₂H), 1674 (C=O); δ_{H} (200 MHz, d_6 -DMSO) 2.60 (s, 3H, -CH₃), 7.36-7.43 (t, 1H, Ar-6H, $J = 7.6$), 7.67-7.71 (d, 1H, Ar-7H, $J = 7.2$), 7.95-7.99 (d, 1H, Ar-5H, $J = 6.9$), 8.03-8.07 (d, 2H, Ph-2'/6'H, $J = 8.5$), 8.27-8.31 (d, 2H, Ph-3'/5'H, $J = 8.5$), 12.7 (s, 1H, -OH), 13.3 (br s, 1H, -NH); m/z (EI) 280 (M⁺, 100%), 235 ([M-CO₂H]⁺), 77 (Ph⁺).

73 8-Methoxy-2-(4'-carboxamidophenyl)quinazolin-4-[3H]-one

8-Methoxy-2-(4'-carboxyphenyl)quinazolin-4-[3H]-one **71** (0.25 g, 0.8 mmol) was suspended in dry THF (20 ml). Thionyl chloride (0.09 ml, 1.3 mmol) and 4 drops dry DMF were added and the reaction mixture was stirred at room temperature for 24 hours. TLC indicated the presence of starting

material and again, thionyl chloride was added (0.09 ml, 1.3 mmol). On completion of the reaction, the mixture was carefully added to a solution of conc. aq. ammonia (10 ml) and stirred at room temperature for 1 hour. The volatiles were removed under reduced pressure to leave the crude product which was collected by filtration and washed with water. The product was purified by column chromatography, using DCM:MeOH (95:5) as eluent to give a cream product (0.08 g, 0.3 mmol, 31%).

$\nu_{\max}/\text{cm}^{-1}$ 3396 and 3201 (1° amide NH), 2839 (OCH₃), 1672 and 1597 (C=O); δ_{H} (200 MHz, *d*₆-DMSO) 4.06 (s, 3H, -OCH₃), 7.47-7.60 (m, 2H, Ar-6/7H), 7.66 (s, 1H, -NH), 7.80-7.84 (d, 1H, Ar-5H, *J* = 7.6), 8.11-8.15 (d, 2H, Ph-2'/6'H, *J* = 7.8), 8.26, (s, 1H, -NH), 8.34-8.39 (d, 2H, Ph-3'/5'H, *J* = 8.4), 12.8 (br s, 1H, -NH); *m/z* (EI) 294 ([M-H]⁺, 96%), 277 ([M-H₂O]⁺).

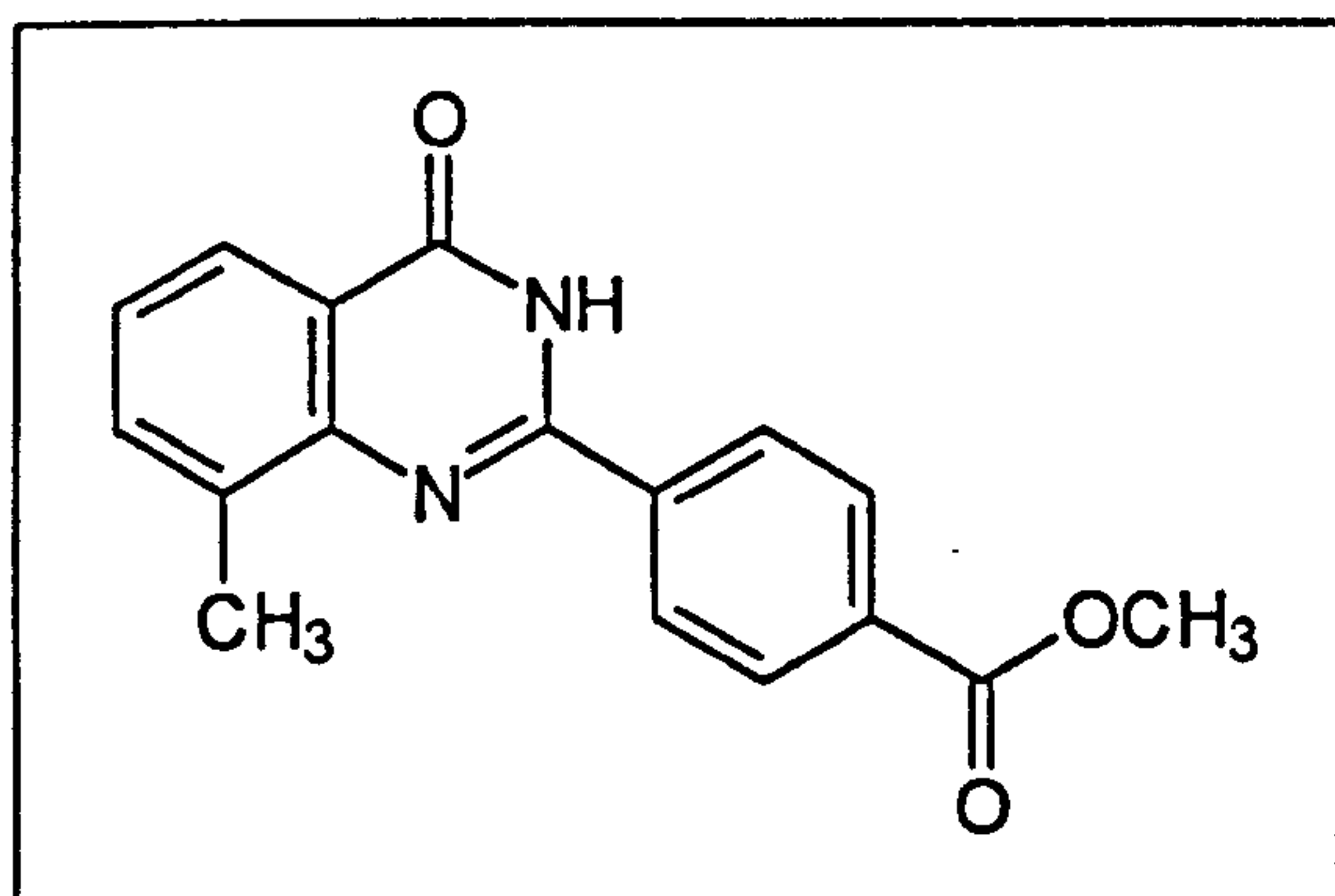
74 8-Methyl-2-(4'-carboxamidophenyl)quinazolin-4-[3H]-one

Prepared in a similar way to compound **73** from 8-methyl-2-(4'-carboxyphenyl)quinazolin-4-[3H]-one **72** (0.2 g, 0.7 mmol). The product was purified by column chromatography using DCM:MeOH (95:5) as eluent to give a cream product (0.05 g, 0.2 mmol, 25%).

$\nu_{\max}/\text{cm}^{-1}$ 3448 and 3200 (1° amide NH), 2921 (CH₃), 1680 (C=O); δ_{H} (200 MHz, *d*₆-DMSO) 2.74 (s, 3H, -CH₃), 7.48-7.56 (t, 1H, Ar-6H, *J* = 7.7), 7.67 (br s, 1H, -NH), 7.80-7.84 (d, 1H,

Ar-7H, $J = 7.2$), 8.09-8.11 (m, 3H, Ar-5H and Ph-2'/6'H, $J = 8.3$), 8.38-8.42 (d, 2H, Ph-3'/5'H, $J = 8.3$), 12.7 (br s, 1H, -NH); m/z (EI) 279 (M^+ , 100%), 235 ($[M-CONH_2]^+$), 77 (Ph^+).

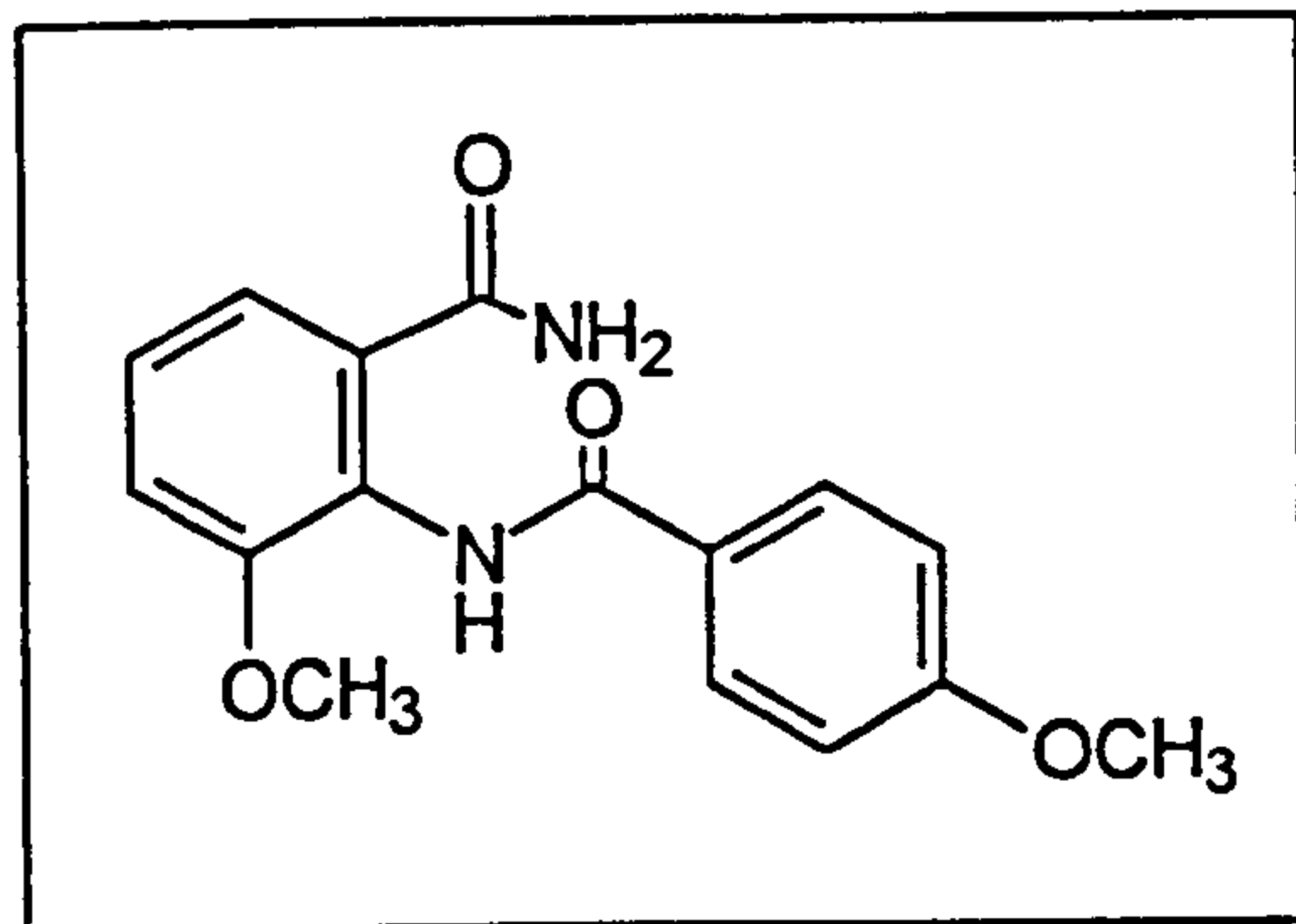
76 8-Methyl-2-(4'-methoxycarbonylphenyl)quinazolin-4-[3H]-one



8-Methyl-2-(4'-carboxyphenyl)quinazolin-4-[3H]-one **72** (0.2 g, 0.7 mmol) was suspended in dry THF (20 ml). Thionyl chloride (0.09 ml, 1.3 mmol) and 2 drops dry DMF were added and the reaction mixture was stirred at room temperature. When TLC indicated the absence of starting

material, the reaction mixture was added to a stirred solution of dry methanol (10 ml). The solvent was removed under reduced pressure to leave a cream solid which was purified by column chromatography, using EtOAc:petrol 40/60 (2:8) as eluent (0.06 g, 0.2 mmol, 24%).

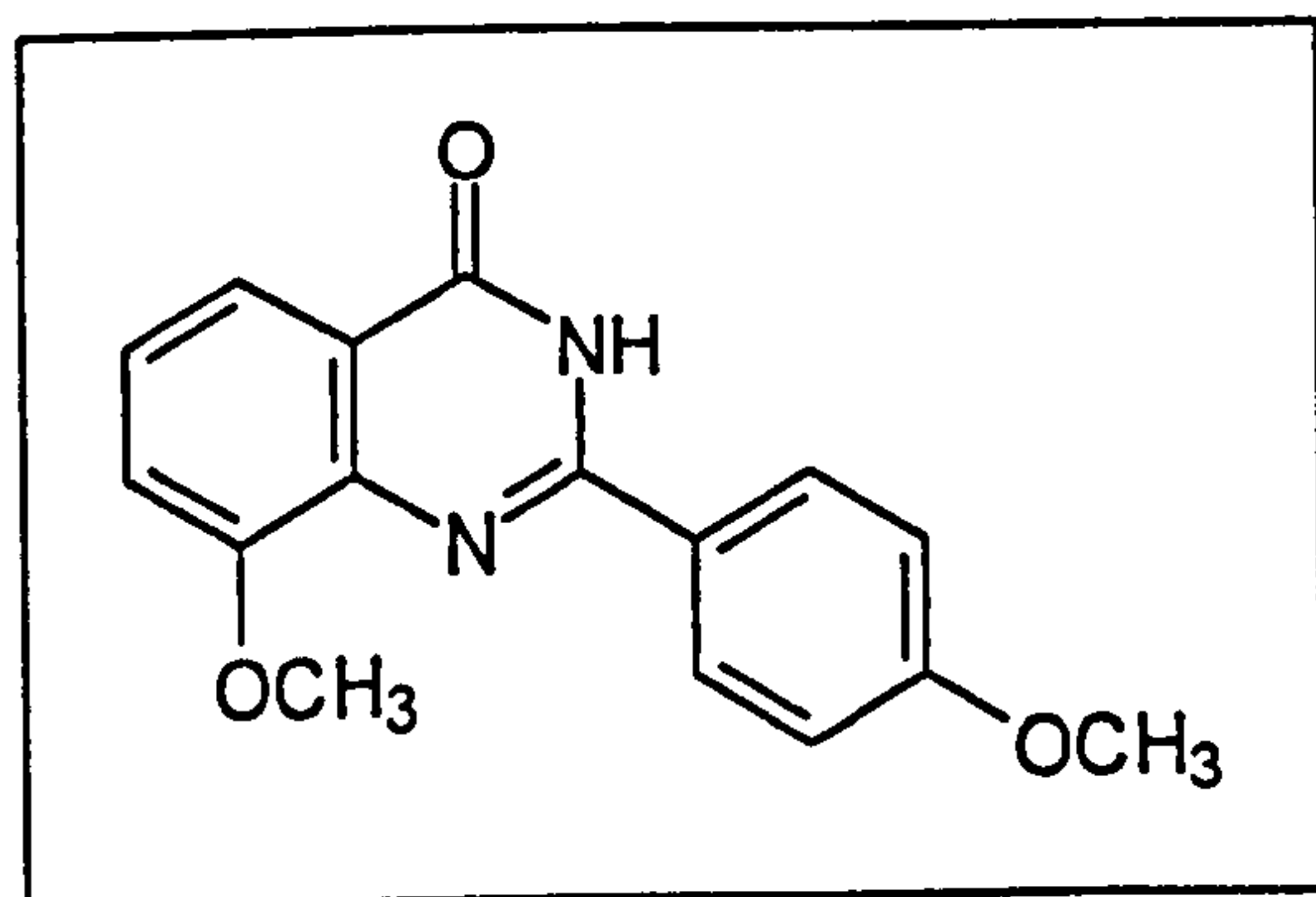
Anal. found C 69.05, H 4.68, N 9.45, $C_{17}H_{14}N_2O_3$ requires C 69.37, H 4.80, N 9.52%; ν_{max}/cm^{-1} 3167 (2° amide NH), 2955 and 2928 (CH_3), 1734 (CO-O), 1681 (C=O); δ_H (200 MHz, d_6 -DMSO) 2.73 (s, 3H, CH_3), 4.00 (s, 3H, OCH_3), 7.49-7.57 (t, 1H, Ar-6H, $J = 7.6$), 7.80-7.84 (d, 1H, Ar-7H, $J = 7.2$), 8.08-8.12 (d, 1H, Ar-5H, $J = 7.9$), 8.18-8.22 (d, 2H, Ph-2'/6'H, $J = 8.4$), 8.43-8.47 (d, 2H, Ph-3'/5'H, $J = 8.4$); m/z (EI) 294 (M^+ , 100%), 235 ($[M-CO_2CH_3]^+$), 77 (Ph^+).

82 3-Methoxy-2-*N*-(4'-methoxybenzoyl)aminobenzamide

Prepared according to Standard Procedure A from 3-methoxy-2-aminobenzamide **41** (0.5 g, 3.0 mmol) and 4-methoxybenzoyl chloride (0.5 ml, 3.3 mmol), in the presence of 4-dimethylaminopyridine (18.4 mg, 0.2 mmol).

The product was recrystallised from methanol/water to give a white crystalline solid (0.43 g, 1.4 mmol, 47%), m.p. 179-181 °C.

Anal. found C 63.92, H 5.15, N 9.02, $C_{16}H_{16}N_2O_4$ requires C 63.99, H 5.37, N 9.33%; ν_{\max} / cm^{-1} 3382 (NH), 3321 (1° amide NH), 3181 (br, 1° amide NH), 2838 (OCH₃), 1673 (C=O); δ_{H} (200 MHz, d_6 -DMSO) 3.88 (s, 3H, -OCH₃), 3.94 (s, 3H, -OCH₃), 7.13-7.17 (d, 2H, Ph-3'/5'H, $J = 8.9$), 7.23-7.31 (m, 3H, Ar-4/5/H), 7.39-7.43 (d, 1H, Ar-6H, $J = 7.7$), 7.49 (br s, 1H, -NH), 7.62 (br s, 1H, -NH), 8.01-8.05 (d, 2H, Ph-2'/6'H, $J = 8.8$), 9.73 (s, 1H, -NH); m/z (EI) 300 (M^+ , 13.7%), 282 ($[M-H_2O]^+$), 253 ($[M-OCH_3]^+$), 149 ($[M-NHCOPhOCH_2]^+$), 135 ($[M-OCH_3 \text{ and } Ph-OCH_2]^+$), 77 (Ph^+).

84 8-Methoxy-2-(4'-methoxyphenyl)quinazolin-4-[3H]-one

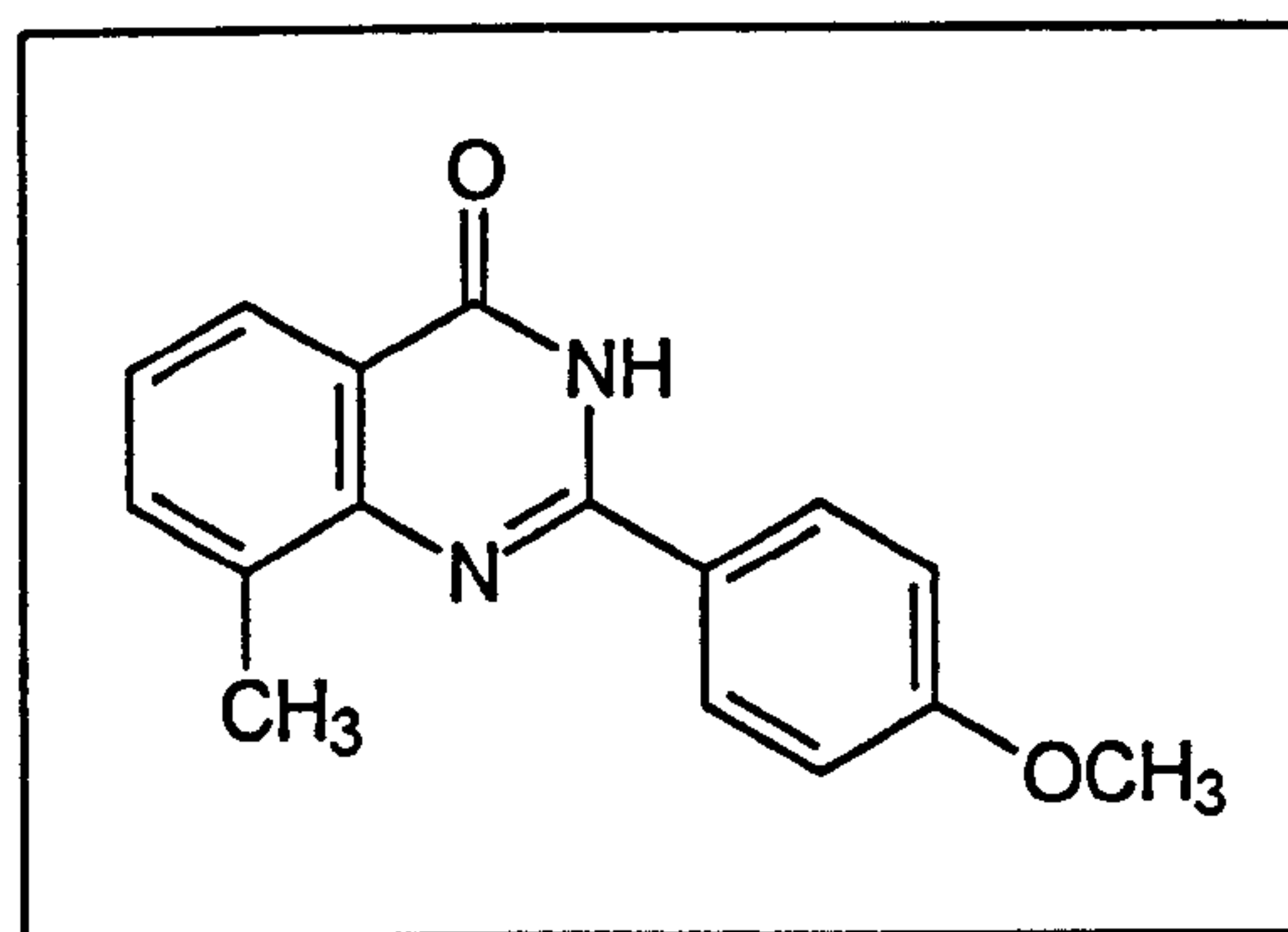
Prepared according to Standard Procedure B from 3-methoxy-2-*N*-(4'-methoxybenzoyl)amino benzamide **82** (0.25 g, 0.8 mmol), in 10% NaOH solution (40 ml) at reflux (2 hours). The product was isolated as white solid after recrystallisation from methanol/water (0.15 g, 0.5 mmol, 63%), m.p.

226-228 °C.

Anal. found C 67.57, H 4.50, N 9.81. $C_{16}H_{13}N_2O_3 \cdot 0.1 \text{ mol. } CH_3OH$ requires C 67.73, H 5.08, N 9.81%; ν_{\max} / cm^{-1} 3174 (2° amide NH), 2837 (OCH₃), 1667 (C=O); δ_{H} (200 MHz, d_6 -DMSO) 3.94 (s, 3H, -OCH₃), 4.04 (s, 3H, -OCH₃), 7.17-7.21 (d, 2H, Ph-3'/5'H, $J = 9.0$), 7.42-

7.55 (m, 2H, Ar-6/7H), 7.76-7.81 (dd, 1H, Ar-5H, $J = 2.1, 7.3$), 8.26-8.31 (d, 2H, Ph-2'/6'H, $J = 9.0$); m/z (EI) 282 (M^+ , 76.7%), 253 ($[M-OCH]^+$), 224 ($[M-OCH, OCH]^+$).

85 8-Methyl-2-(4'-methoxyphenyl)quinazolin-4-[3H]-one

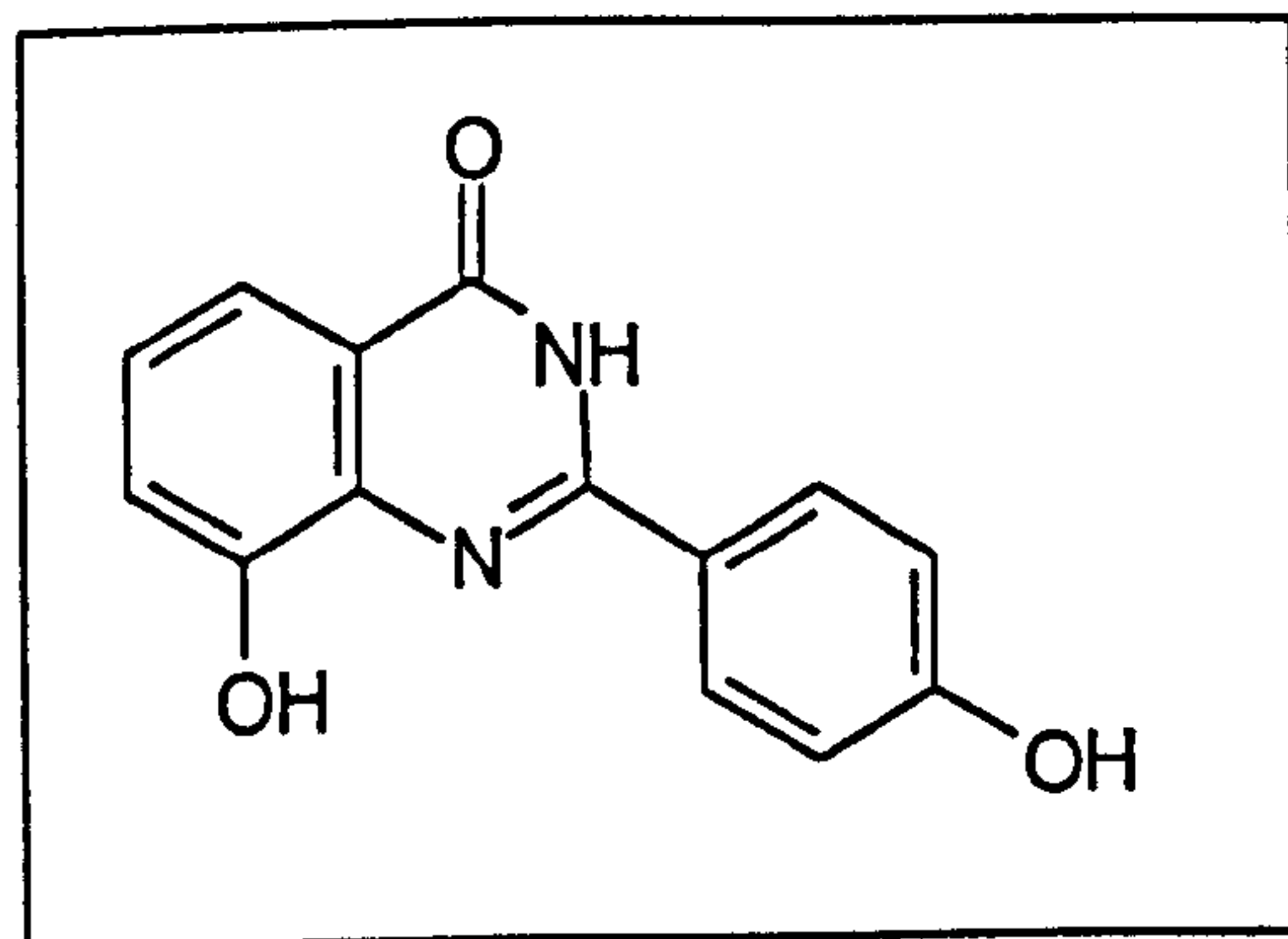


Prepared according to Standard Procedure C from 3-methyl-2-aminobenzamide 44 (0.2 g, 1.3 mmol) and benzoyl chloride (0.22 ml, 1.5 mmol), in the presence of 4-dimethylaminopyridine (8.1 mg, 5 ol. %). The product was obtained as a white crystalline solid after recrystallisation from methanol/water (0.21 g, 0.8

mmol, 58%), m.p. 227-229 °C.

Anal. found C 71.89, H 5.23, N 10.20, $C_{16}H_{14}N_2O_2$ requires C 72.17, H 5.30, N 10.52%; ν_{max}/cm^{-1} 3177 (2° amide NH), 2962 and 2934 (CH_3), 2837 (OCH_3), 1674 ($C=O$); δ_H (200 MHz, d_6 -DMSO) 2.71 (s, 3H, $-CH_3$), 3.95 (s, 3H, $-OCH_3$), 7.18-7.22 (d, 2H, Ph-3'/5'H, $J = 8.9$), 7.42-7.50 (t, 1H, Ar-6H, $J = 7.6$), 7.76-7.79 (d, 1H, Ar-7H, $J = 6.6$), 8.05-8.09 (d, 1H, Ar-5H, $J = 7.2$), 12.63 (s, 1H, $-NH$); m/z (EI) 266 (M^+ , 100%), 251 ($[M-CH_3]^+$), 236 ($[M-OCH_2]^+$), 105 ($PhC=O^+$), 77 (Ph^+).

86 8-Hydroxy-2-(4'-hydroxyphenyl)quinazolin-4-[3H]-one

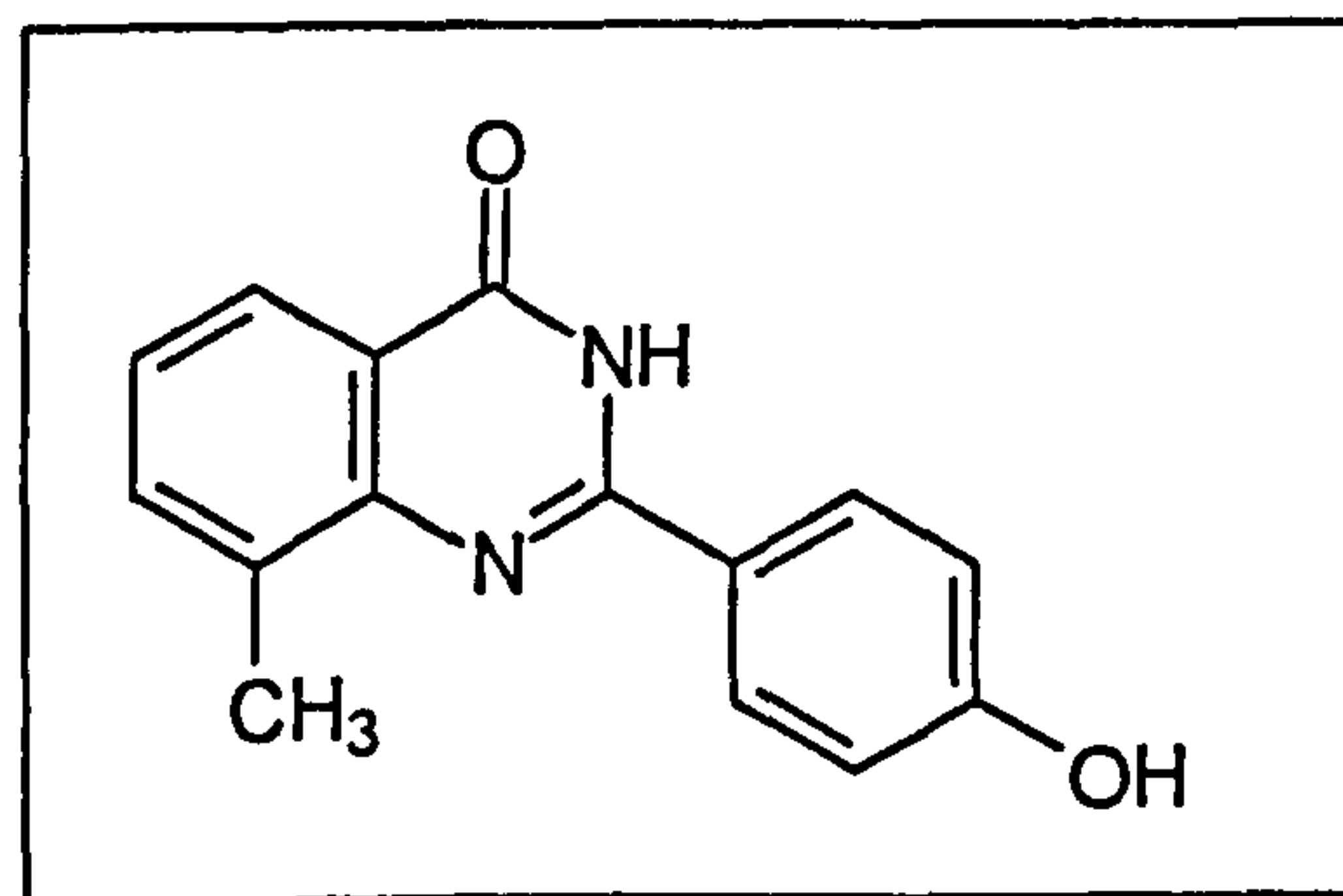


Prepared according to Standard Procedure D, from 8-methoxy-2-(4'-methoxyphenyl)quinazolin-4-[3H]-one 84 (0.2 g, 0.71 mmol). The product was recrystallised from methanol to give a cream crystalline solid (0.08 g, 0.33 mmol, 47%), m.p. 288-290 °C.

Anal. found C 66.20, 66.09, H 4.02, 4.11, N 10.98, 10.99, $C_{14}H_{10}N_2O_3$ requires C 66.13, H 3.96, N 11.02%; ν_{max}/cm^{-1} 3374 (br, OH), 1684 ($C=O$); δ_H (200 MHz, d_6 -DMSO) 6.95-7.00

(d, 2H, Ph-2'/6'H, $J = 8.8$), 7.26-7.31 (dd, 1H, Ar-7H, $J = 1.6, 7.8$), 7.34-7.42 (t, 1H, Ar-6H, $J = 7.8$), 7.62-7.66 (dd, 1H, Ar-5H, $J = 1.7, 7.7$), 8.38-8.42 (d, 2H, Ph-3'/5'H, $J = 8.8$), 9.6 (br s, 1H, -OH), 12.3 (br s, 1H, -NH); m/z (EI) 254 (M^+ , 42.4%)

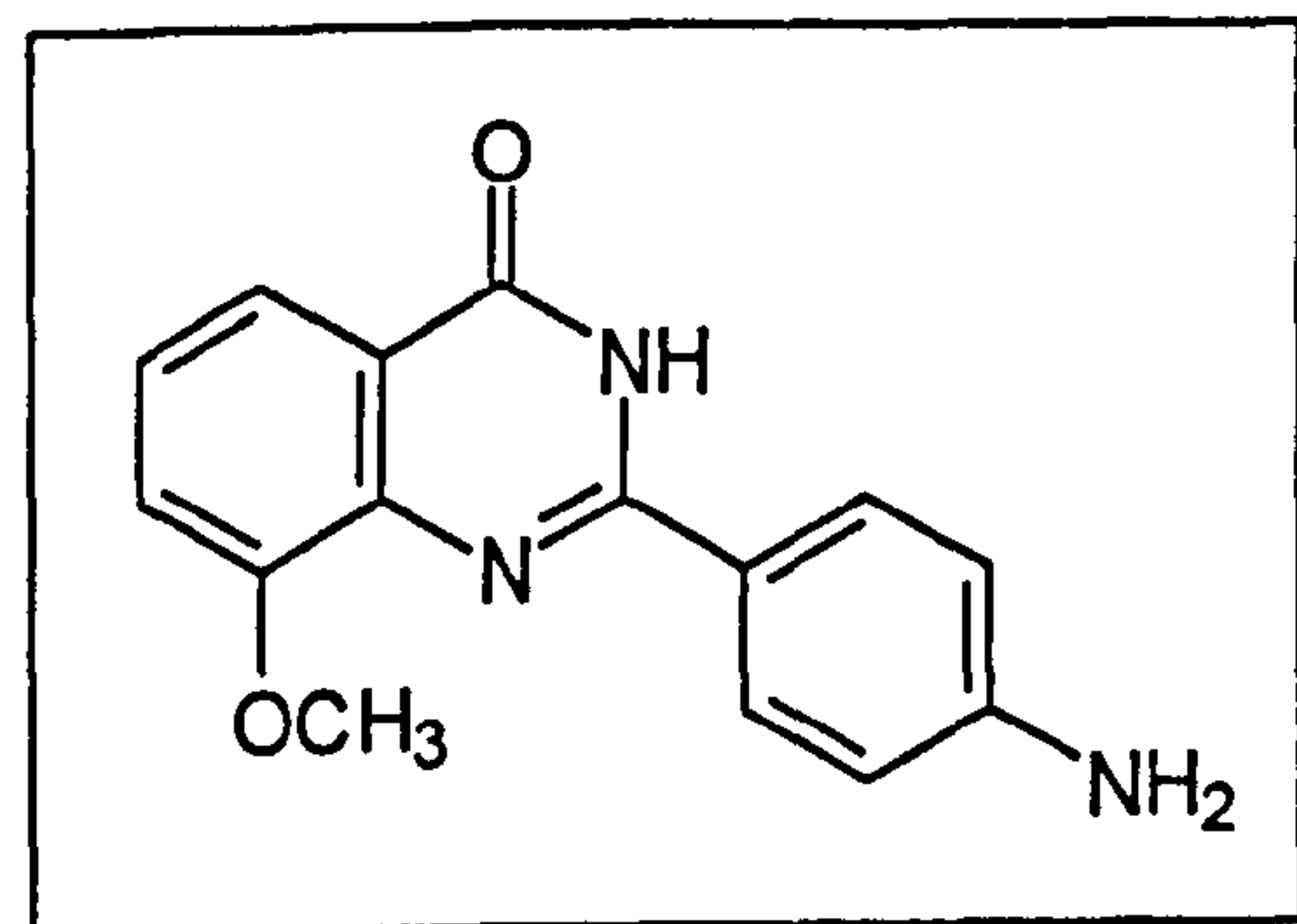
87 8-Methyl-2-(4'-hydroxyphenyl)quinazolin-4-[3H]-one



Prepared according to Standard Procedure D from 8-methyl-2-(4'-methoxyphenyl)quinazolin-4-[3H]-one 85 (0.2 g, 0.75 mmol). The product was recrystallised from methanol/water to give cream crystals (0.14 g, 0.57 mmol, 76%), m.p. 258-261 °C.

Anal. found C 71.34, H 4.86, N 10.82, $C_{15}H_{12}N_2O_2$ requires C 71.41, H 4.79, N 11.11%; ν_{max}/cm^{-1} 3246 (br, OH), 3169 (br, 2° amide NH), 2949 (CH_3), 1680 (C=O); δ_H (200 MHz, d_6 -DMSO) 2.69 (s, 3H, $-CH_3$), 6.97-7.02 (dd, 2H, Ph-2'/6'H, $J = 1.9, 6.9$), 7.39-7.47 (t, 1H, Ar-6H, $J = 7.6$), 7.74-7.77 (d, 1H, Ar-7H, $J = 6.7$), 8.03-8.07 (dd, 1H, Ar-5H, $J = 1.1, 8.0$), 8.19-8.24 (d, 2H, Ph-3'/5'H, $J = 2.0, 6.7$), 10.3 (br s, 1H, -OH), 12.4 (br s, 1H, -NH); m/z (EI) 252 (M^+ , 100%), 209 ($[M-CH_3CO]^+$), 77 (Ph^+).

88 8-Methoxy-2-(4'-aminophenyl)quinazolin-4-[3H]-one

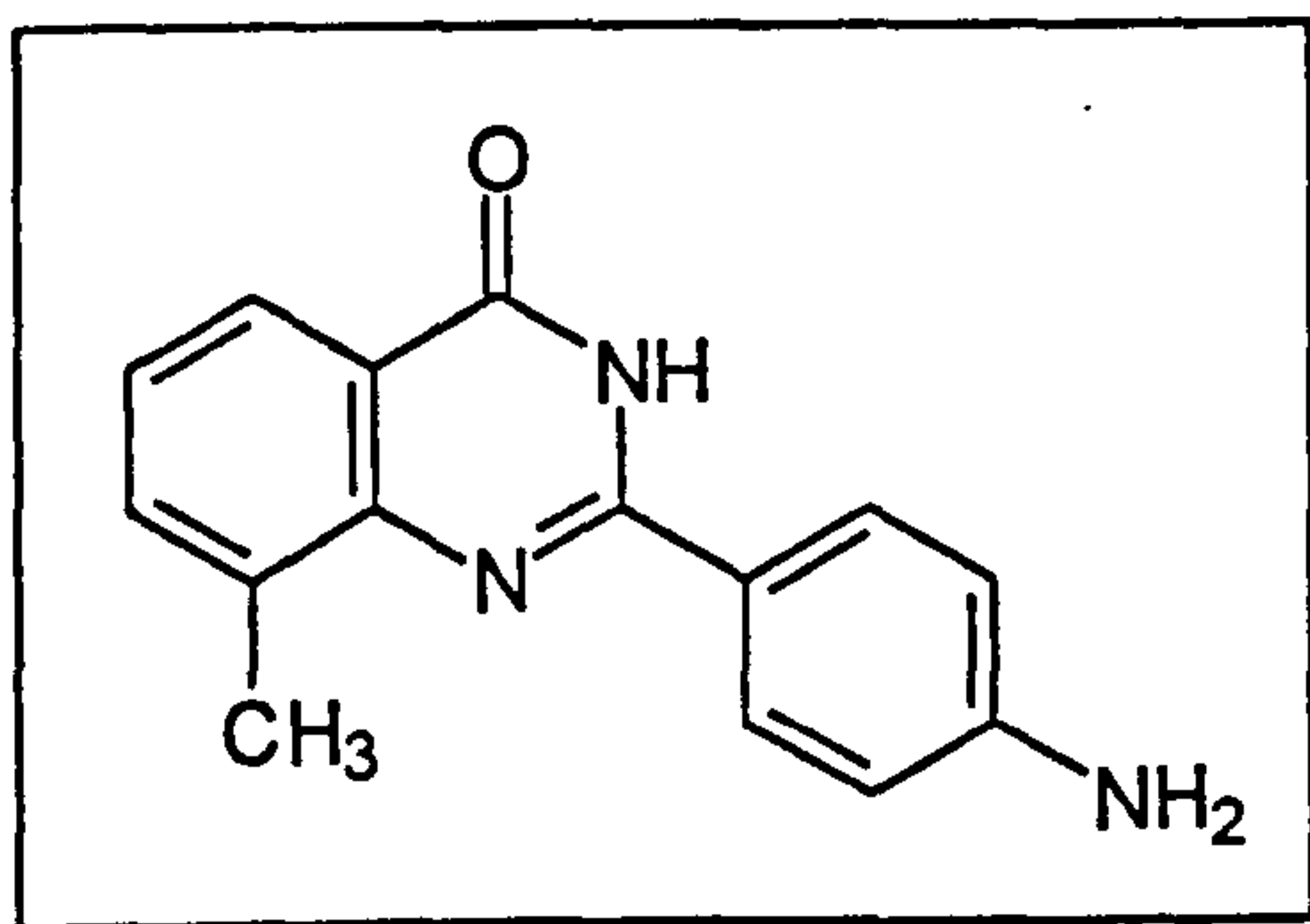


8-Methoxy-2-(4'-nitrophenyl)quinazolin-4-[3H]-one 55 (0.1 g, 0.3 mmol) was suspended in methanol (100 ml) with 10% palladium-carbon catalyst (50 mg). The reaction vessel was placed under an atmosphere of hydrogen, at ambient temperature and pressure, until no further hydrogen absorption was observed. The catalyst

was removed by filtration through Celite to leave a colourless solution. The solvent was removed under vacuum to yield the title compound, which was recrystallised from methanol/water to give off-white crystals (0.04 g, 0.16 mmol, 46%), m.p. 263-265 °C.

Anal. found C 65.30, H 4.89, N 15.04, $C_{15}H_{13}N_3O_2 \cdot 0.4$ mol. H_2O requires C 65.63, H 5.07, N 15.31%; $\nu_{\max}/\text{cm}^{-1}$ 3446 (NH), 3337 (NH), 2836 (OCH_3), 1671 ($\text{C}=\text{O}$); δ_{H} (200 MHz, d_6 -DMSO) 4.02 (s, 3H, $-\text{OCH}_3$), 5.93 (s, 2H, $-\text{NH}_2$), 6.71-6.75 (d, 2H, Ph-2'/6'H, $J = 8.7$), 7.41-7.44 (m, 2H, Ar-6/7H), 7.72-7.77 (dd, 1H, Ar-5H, $J = 3.1, 6.3$), 8.03-8.08 (d, 2H, Ph-3'/5'H, $J = 8.7$), 12.1 (s, 1H, $-\text{NH}$); m/z (EI) 267 (M^+ , 88%), 237 ($[M-\text{OCH}_2]^+$), 92 (PhNH^+).

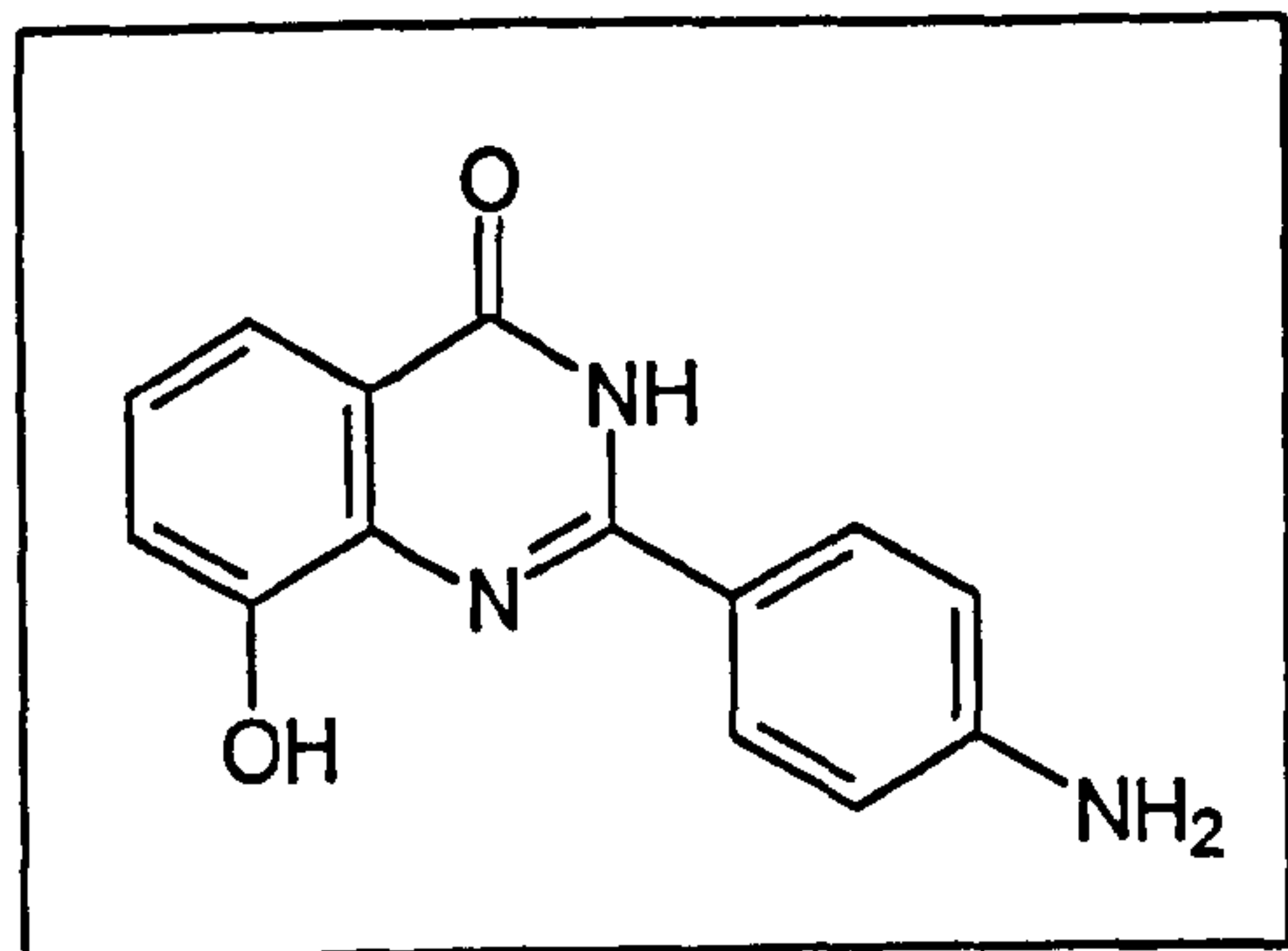
89 8-Methyl-2-(4'-aminophenyl)quinazolin-4-[3H]-one



Prepared in a similar way to compound 88 from 8-methyl-2-(4'-nitrophenyl)quinazolin-4-[3H]-one 57 (50 mg, 0.18 mmol). The product was recrystallised from methanol/water to give white crystals (0.02 g, 0.09 mmol, 52%), m.p. 254-256 °C.

Anal. found C 71.62, H 4.85, N 16.74, $C_{15}H_{13}N_3O$ requires C 71.69, H 5.21, N 16.73%; $\nu_{\max}/\text{cm}^{-1}$ 3432 (2° amide NH), 3397 (2° amide NH), , 2950 and 2918 (CH_3), 1663 ($\text{C}=\text{O}$); δ_{H} (200 MHz, d_6 -DMSO) 2.67 (s, 3H, $-\text{CH}_3$), 5.96 (s, 2H, $-\text{NH}_2$), 6.72-6.77 (d, 2H, Ph-3'/5'H, $J = 8.7$), 7.35-7.43 (t, 1H, Ar-6H, $J = 7.6$), 7.71-7.75 (d, 1H, Ar-7H, $J = 6.7$), 8.01-8.03 (d, 1H, Ar-5H, $J = 7.9$), 8.09-8.13 (d, 2H, Ph-2'/6'H, $J = 8.7$), 12.1 (s, 1H, $-\text{NH}$); m/z (EI) 251 (M^+ , 100%), 236 ($[M-\text{CH}_3]^+$), 92 (PhNH^+).

90 8-Hydroxy-2-(4'-aminophenyl)quinazolin-4-[3H]-one

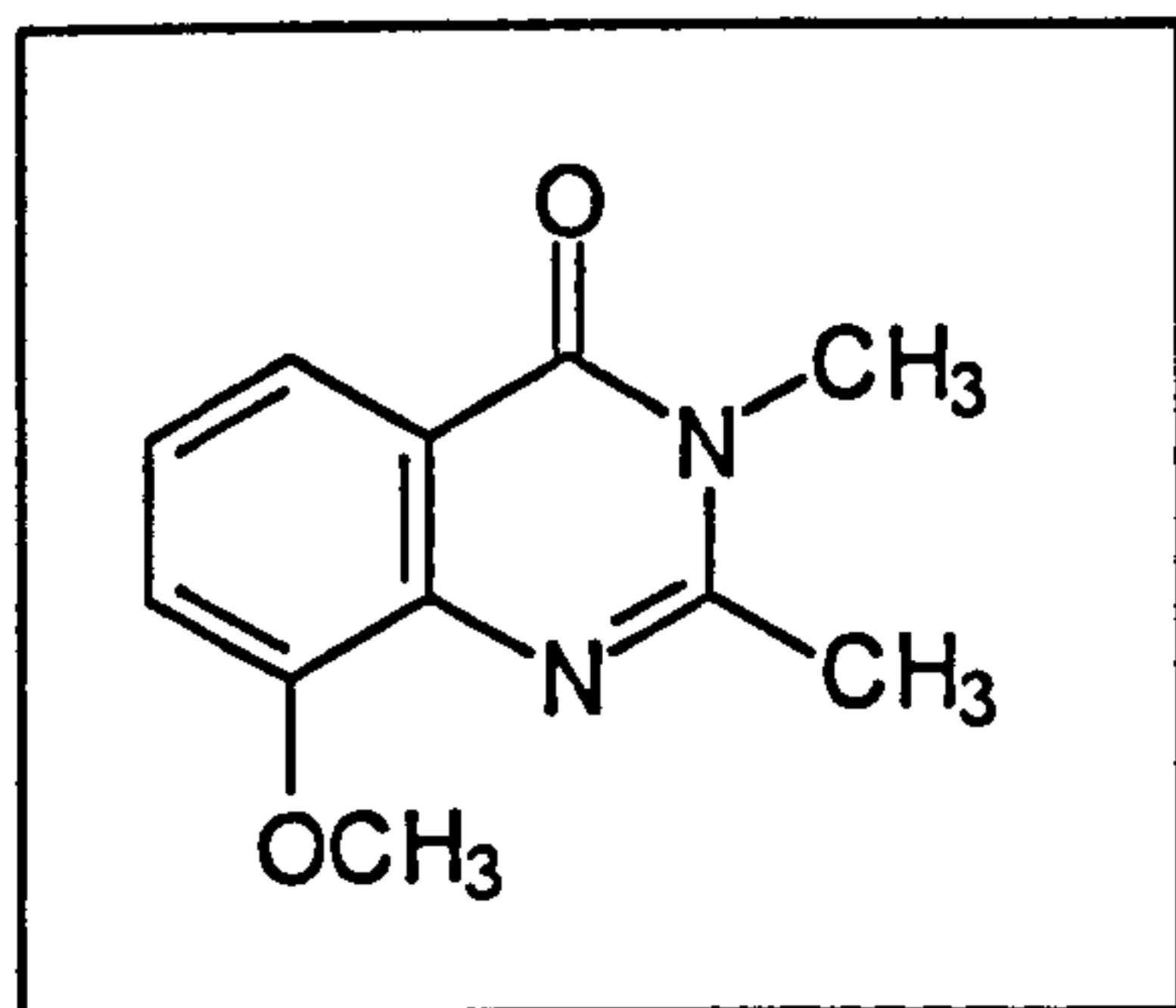


Prepared in a similar way to compound 88 from 8-hydroxy-2-(4'-nitrophenyl)quinazolin-4-[3H]-one 38 (50 mg, 0.18 mmol). The product was recrystallised from methanol/water to give cream/pale brown crystals (25.7 mg, 0.1 mmol, 57%), m.p. 274-276 °C.

Anal. found C 65.49, H 4.30, N 15.95, $C_{14}H_{11}N_3O_2 \cdot 0.2$ mol. H_2O requires C 65.46, H 4.47, N 16.36%; $\nu_{\max}/\text{cm}^{-1}$ 3452 (OH), 3432 (NH), 3359 (NH),

1592 (C=O); δ_{H} (200 MHz, d_6 -DMSO) 5.94 (s, 2H, -NH₂), 6.72-6.76 (d, 2H, Ph-3'/5'H, J = 8.6), 7.25-7.38 (m, 2H, Ar-6/7H), 7.61-7.65 (d, 1H, Ar-5H, J = 7.5), 8.26-8.31 (d, 2H, Ph-2'/6'H, J = 8.5), 9.40 (br s, 1H, -OH), 12.15 (br s, 1H, -NH); m/z (EI) 253 (M⁺, 100%), 92 (PhNH⁺).

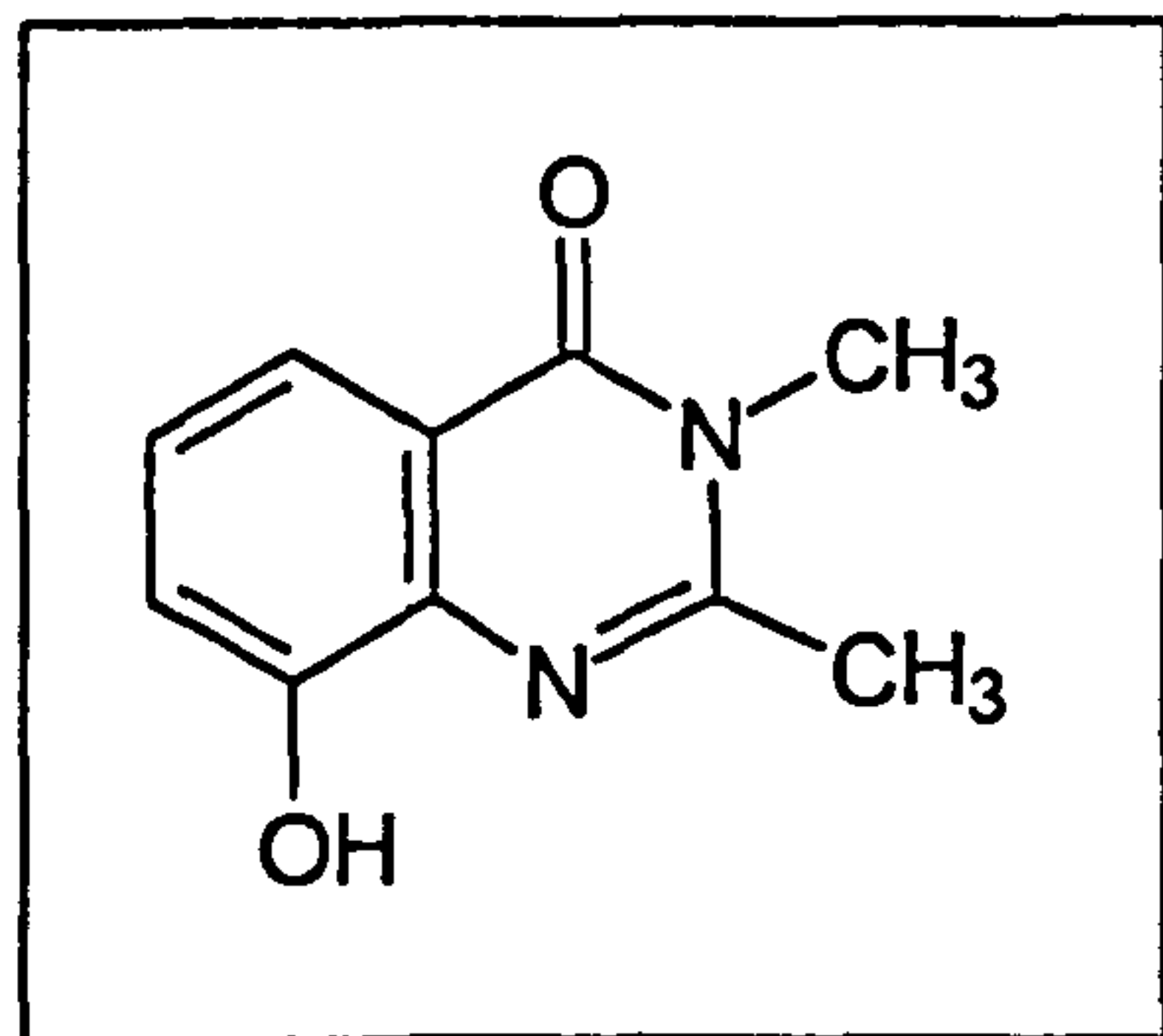
91 8-Methoxy-3-*N*-methyl-2-methylquinazolin-4-[3H]-one²⁶³



8-Methoxy-2-methylquinazolin-4-[3H]-one 46 (0.5 g, 2.6 mmol) was suspended in dry acetonitrile (60 ml) with potassium carbonate (0.36 g, 2.6 mmol) and methyl iodide (0.16 ml, 2.6 mmol) and the reaction mixture was refluxed for 34 hours. The solvent was removed under vacuum to leave a cream solid which was resuspended in water and extracted into

EtOAc. The organic layers were combined, dried (MgSO₄) and filtered. The solvent was removed under vacuum to leave a pale yellow residue, which was recrystallised from EtOAc/petrol 40/60 to give the product as pale yellow crystals (0.3 g, 1.47 mmol, 56%), m.p. 133-136 °C.

Anal. found C 59.76, H 6.32, N 12.60, C₁₁H₁₂N₂O₂ requires C 59.44, H 6.35, N 12.61%; $\nu_{\text{max}}/\text{cm}^{-1}$ 2946 (CH₃), 2841 (OCH₃), 1683 and 1659 (3° amide), 1599 (C=O); δ_{H} (200 MHz, d_6 -DMSO) 2.66 (s, 3H, -CH₃), 3.62 (s, 3H, N-CH₃), 3.98 (s, 3H, -OCH₃), 7.37-7.42 (d, 1H, Ar-7H, J = 6.9), 7.44-7.52 (t, 1H, Ar-6H, J = 7.9), 7.71-7.75 (dd, 1H, Ar-5H, J = 7.7); m/z (EI) 204 (M⁺, 78.7%), 203 ([M-H]⁺), 174 ([M-CH₃CH₃]⁺), 146 ([M-CH₃CH₃OC]⁺).

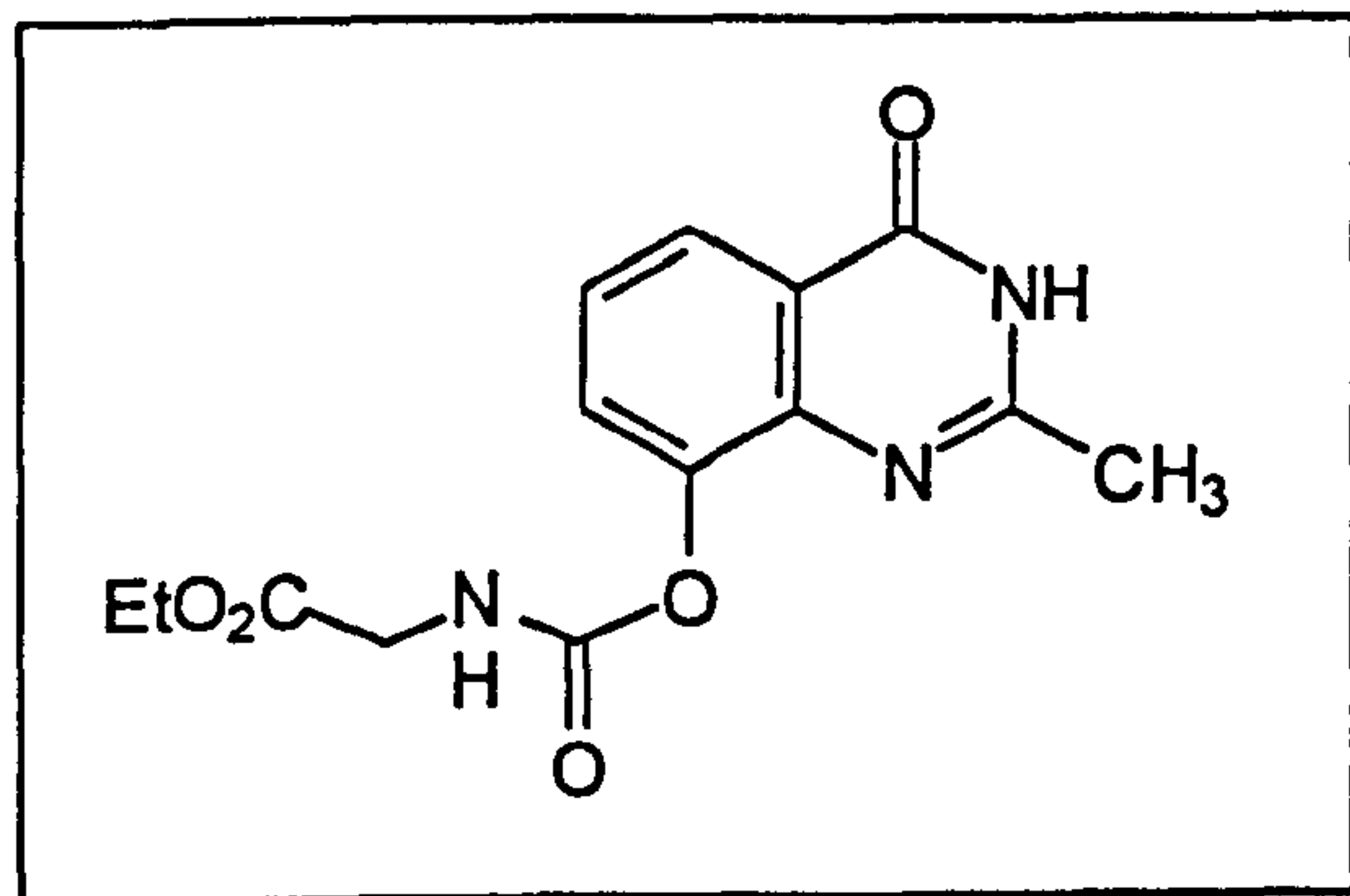
92 8-Hydroxy-3-*N*-methyl-2-methylquinazolin-4-[3*H*]-one

Prepared according to Standard Procedure D from 8-methoxy-3-*N*-methyl-2-methylquinazolin-4-[3*H*]-one 91 (0.2 g, 0.98 mmol).

Crude yield : 0.18 g, 0.94 mmol, 96%. The product (0.155 g) was purified by column chromatography, using DCM:MeOH (98:2) as eluent, to give a white solid (0.106 g, 0.56 mmol,

57%), m.p. 145-147 °C.

Anal. found C 62.46, 62.48, H 5.28, 5.24, N 14.26, 14.37, $C_{10}H_{10}N_2O_2 \cdot 0.1 \text{ mol. H}_2\text{O}$ requires C 62.55, H 5.35, N 14.59%; $\nu_{\text{max}}/\text{cm}^{-1}$ 3388 (br, OH), 3028 (CH_3), 1689 (3° amide), 1599 ($\text{C}=\text{O}$); δ_{H} (200 MHz, d_6 -DMSO) 2.71 (s, 3H, $-\text{CH}_3$), 3.63 (s, 3H, $\text{N}-\text{CH}_3$), 7.23-7.27 (dd, 1H, Ar-7H, $J = 1.5, 7.8$), 7.34-7.42 (t, 1H, Ar-6H, $J = 7.8$), 7.59-7.64 (dd, 1H, Ar-5H, $J = 1.5, 7.8$); m/z (EI) 190 (M^+ , 37%),

93 8-*O*-(Glycine carbamate ethyl ester)-2-methylquinazolin-4-[3*H*]-one

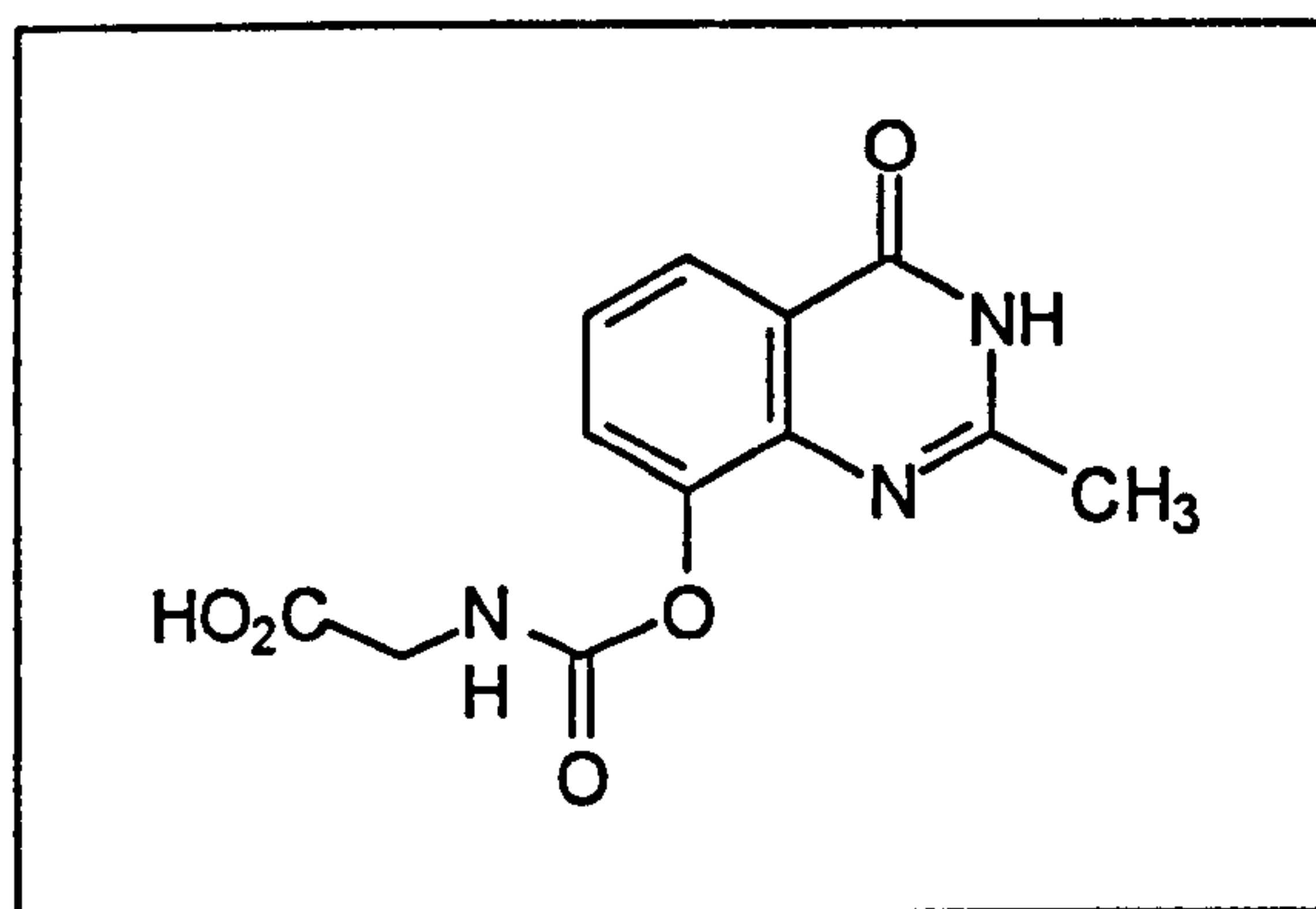
8-Hydroxy-2-methylquinazolin-4-[3*H*]-one 37 (0.2 g, 1.14 mmol) was dissolved in dry THF (40 ml) with dry triethylamine (0.32 ml, 2.27 mmol) and ethyl isocyanatoacetate (0.14 ml, 1.24 mmol). The reaction mixture was stirred under nitrogen for 24 hours and a white precipitate formed. The solvent

was removed under vacuum to leave a white solid, which was redissolved in EtOAc and washed with water. The organic layers were combined, dried (MgSO_4) and filtered. The solvent was removed under vacuum and the crude residue was recrystallised from EtOAc/petrol 40/60 to give a white solid (0.27 g, 0.90 mmol, 79%), m.p. 208-209 °C.

Anal. found C 53.19, H 4.93, N 13.05, $C_{14}H_{15}N_3O_5 \cdot 0.5 \text{ mol. H}_2\text{O}$ requires C 53.45, H 5.13, N 13.37%; $\nu_{\text{max}}/\text{cm}^{-1}$ 3352 and 3301 (br, 2° amide NH), 2908 (CH_3), 1717 ($\text{C}=\text{O}$), 1680 ($\text{C}=\text{O}$);

δ_H (200 MHz, d_6 -DMSO) 1.27-1.35 (t, 3H, $-\text{CH}_3\text{CH}_2$, $J = 7.1$), 2.44 (s, 3H, $-\text{CH}_3$), 3.95-3.98 (d, 2H, $-\text{CH}_2-\text{CO}_2\text{C}_2\text{H}_5$, $J = 6.0$), 4.18-4.28 (q, 2H, $-\text{CH}_3\text{CH}_2\text{OCO}$, $J = 7.1$, 14.2) 7.48-7.63 (m, 2H, Ar-6/7H), 8.02-8.06 (dd, 1H, Ar-5H, $J = 1.6$, 7.7), 8.39-8.44 (t, 1H, $-\text{NH}-\text{CO}$, $J = 6.0$), 12.4 (s, 1H, $-\text{NH}$); m/z (EI) No M^+ found, 176 ($[\text{M}-\text{CO}_2\text{NH}-\text{CH}_2-\text{CO}_2\text{Et}]^+$).

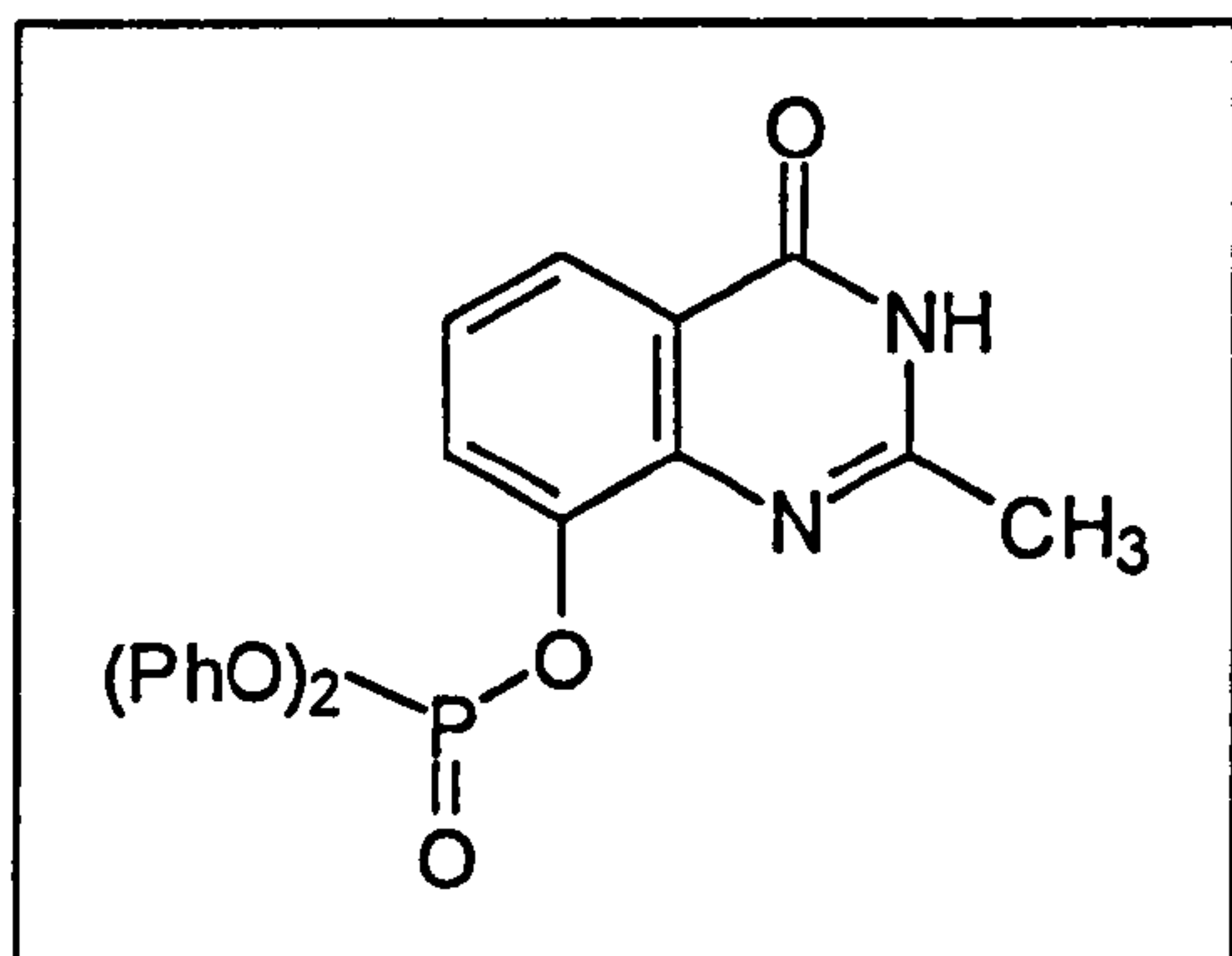
94 8-*O*-(Glycine carbamate)-2-methylquinazolin-4-[3*H*]-one



8-*O*-(Glycine carbamate ethyl ester)-2-methylquinazolin-4-[3*H*]-one 93 (1.0 g, 3.3 mmol) was suspended in dry THF (20 ml). On addition of 0.5 M H_2SO_4 (20 ml) the compound fully dissolved to give a clear, colourless solution, which was stirred at room temperature (12 hours).

The THF was removed under vacuum and the remaining aqueous phase was diluted with an equal volume of water (pH 0.5) whereupon there was slow formation of a white precipitate. The solution was filtered and the white product was collected and dried. The pH of the filtrate was adjusted to 2.0 with aq. NaOH solution (1.0 M). Again a white precipitate formed which was collected by filtration and dried. The combined products were found to be identical by ^1H NMR analysis (0.58 g, 2.1 mmol, 63%), m.p. 222-224 °C.

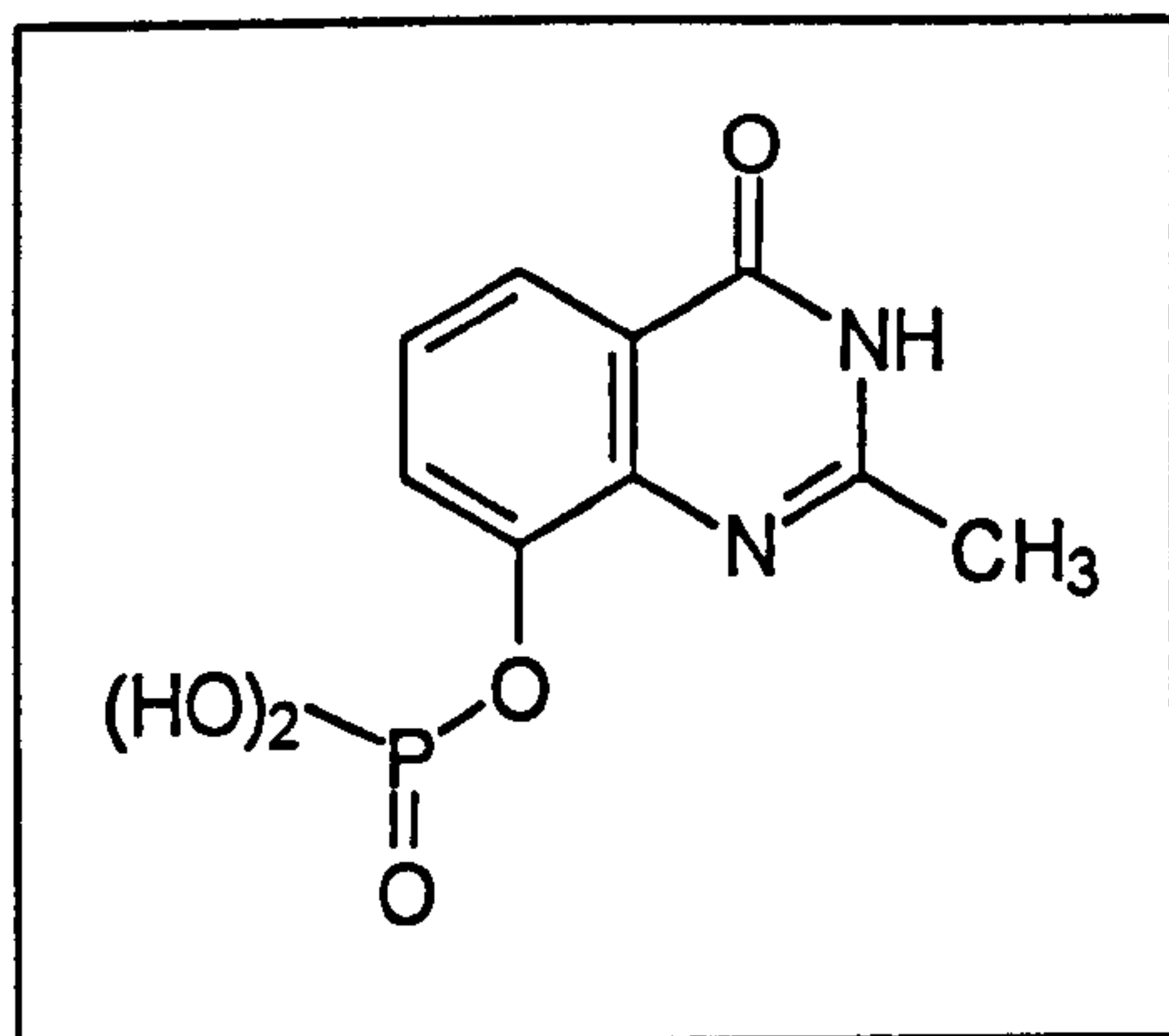
Anal. found C 49.10, H 3.62, N 13.97, $\text{C}_{12}\text{H}_{11}\text{N}_3\text{O}_5 \cdot 0.5\text{mol.HCl}$ requires C 48.78, H 3.92, N 14.22%; $\nu_{\text{max}}/\text{cm}^{-1}$ 3369 (NH), 1760 (CO_2H), 1676 (C=O); δ_H (200 MHz, d_6 -DMSO) 2.44 (s, 3H, $-\text{CH}_3$), 3.86-3.89 (d, 2H, $\text{CH}_2-\text{CO}_2\text{H}$, $J = 6.0$), 7.49-7.65 (m, 2H, Ar-6/7H), 8.03-8.07 (d, 1H, Ar-5H, $J = 7.7$), 8.30-8.36 (t, 1H, $-\text{NH}-\text{CO}$, $J = 6.0$), 12.4 (br s, 1H, $-\text{NH}$); m/z (EI) No M^+ found 176 ($[\text{M}-\text{EtO}_2\text{C}-\text{CH}_2\text{NHCO}_2]^+$).

95 8-(*O*-diphenylphosphoryl)-2-methylquinazolin-4-[3*H*]-one²⁷⁹

8-Hydroxy-2-methylquinazolin-4-[3*H*]-one **37** (0.1 g, 0.57 mmol) was suspended in dry acetonitrile (7 ml), with *N,N*-diisopropylethylamine (0.11 ml, 0.62 mmol) and diphenyl chlorophosphate (0.13 ml, 0.62 mmol). The reaction mixture was stirred under nitrogen at room temperature for 2 hours. Isopropanol (1 ml) was added

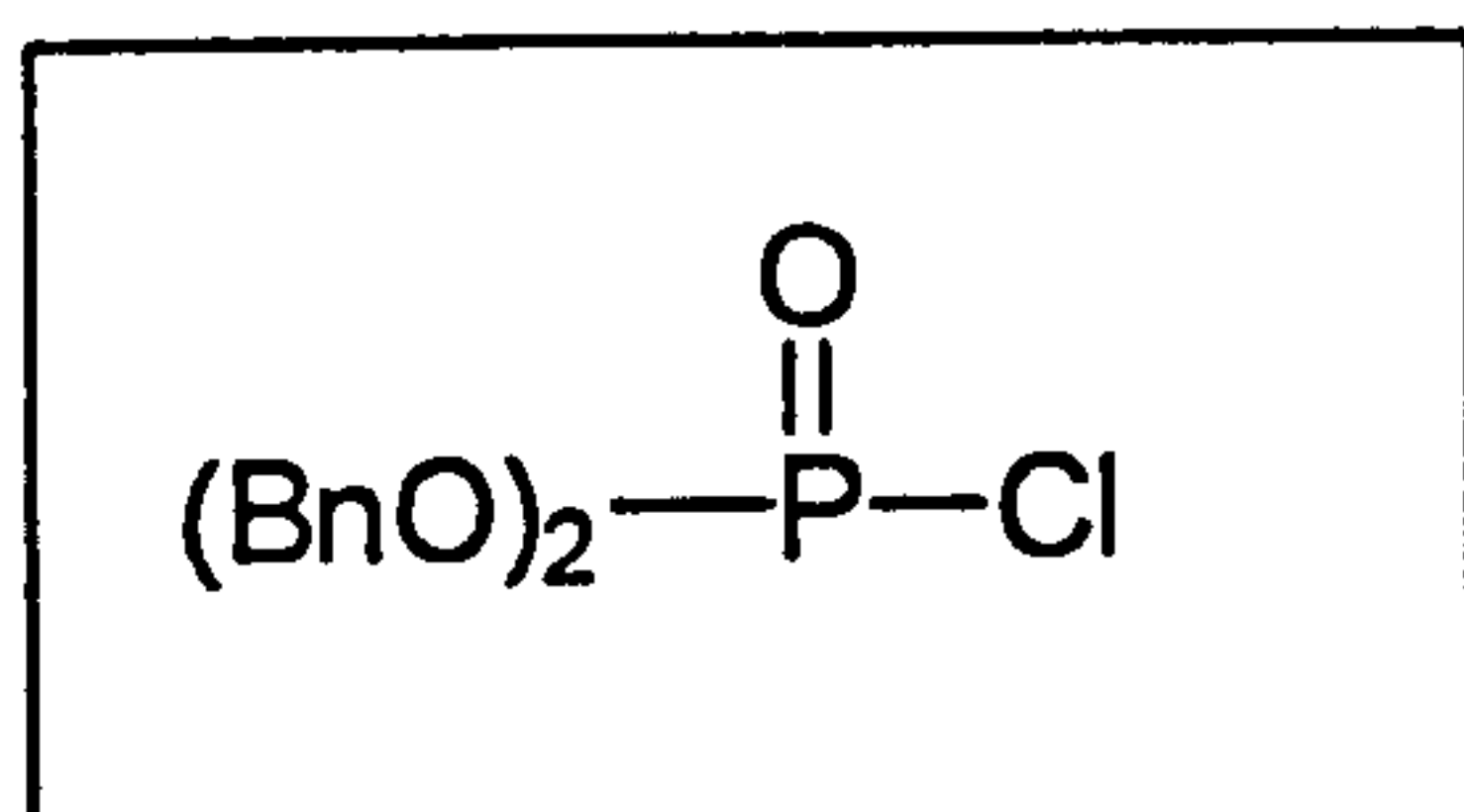
and the solvents were removed under vacuum to leave a white solid, which was redissolved in DCM and washed with water. The organic layer was dried (MgSO₄), filtered and the solvent was removed under vacuum to give a white solid, which was recrystallised from EtOAc/petrol 40/60 (0.108 g, 0.26 mmol, 46%), m.p. 164-165 °C.

Anal. found C 61.52, H 4.27, N 6.72, C₂₁H₁₇N₂O₃P requires C 61.71, H 4.20, N 6.68%; $\nu_{\max}/\text{cm}^{-1}$ 3171 (2° amide NH), 2791 (CH₃), 1674 (C=O), 1181 (P-O-aryl); δ_{H} (200 MHz, *d*₆-DMSO) 2.43 (s, 3H, -CH₃), 7.36-7.61 (m, 11H, Ar-6H, 2 × Ph-2'/3'/4'/5'/6'H), 7.73-7.78 (dd, 1H, Ar-7H), 8.06-8.11 (dd, 1H, Ar-5H), 12.55 (s, 1H, -NH); *m/z* (FAB) 409 (MH⁺, 15%), 315 ([M-PhOH]⁺), 77 (Ph⁺).

96 Attempted Preparation of 8-(*O*-phosphoryl)-2-methylquinazolin-4-[3*H*]-one²⁷⁹

8-*O*-(diphenylphosphoryl)-2-methylquinazolin-4-[3*H*]-one **95** (0.2 g, 0.49 mmol) was dissolved in ethanol (20 ml) containing a suspension of platinum oxide (0.12 g) and the reaction mixture was hydrogenated in a Parr hydrogenator under 45-50 p.s.i. for 4 hours at room temperature. The catalyst was removed by filtration through Celite to give a clear, colourless solution. The

solvent was removed under vacuum to give a pale brown oil, which solidified on drying *in vacuo*. No positive analytical results were obtained for this compound.

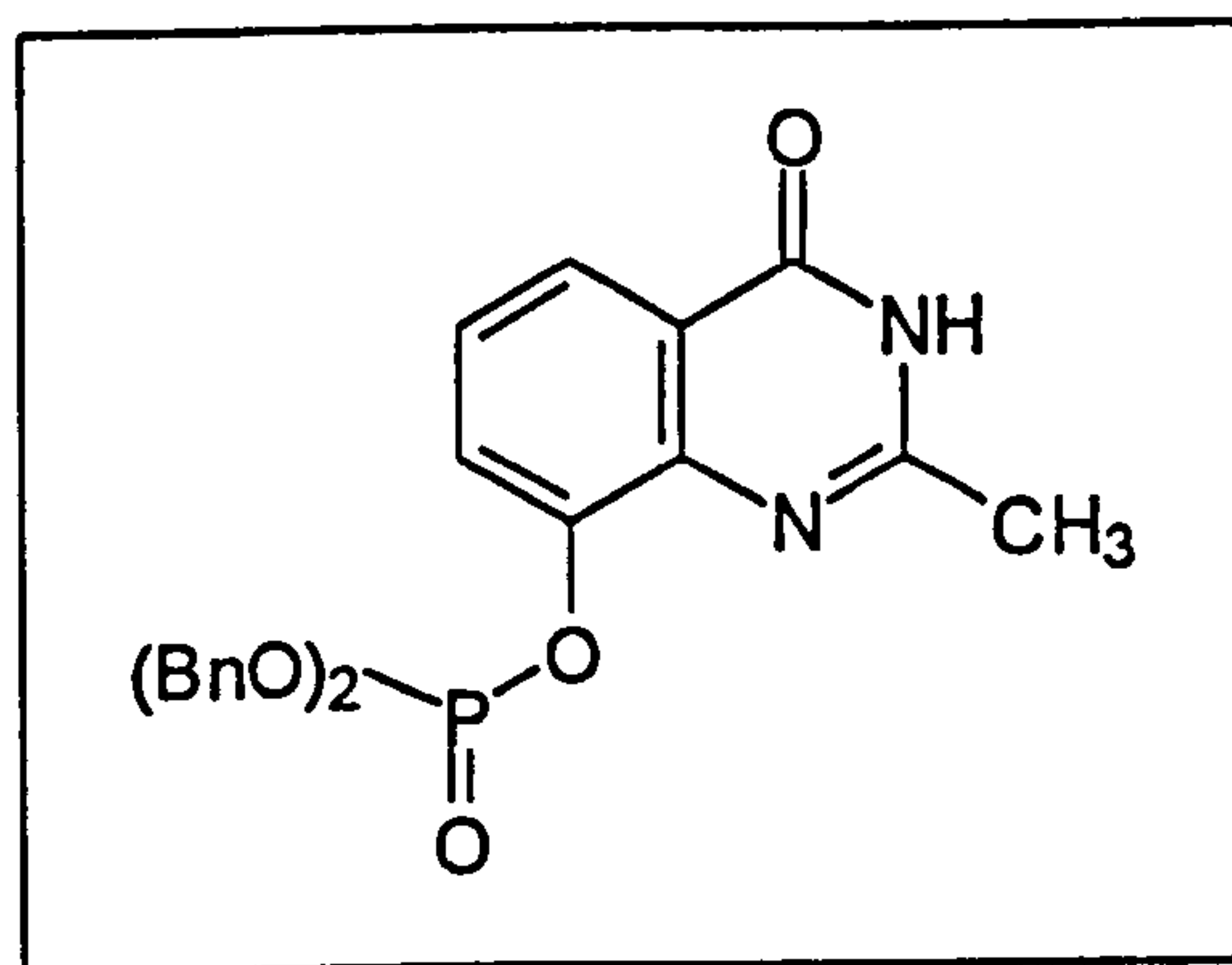
97 Dibenzyl phosphorochloridate²⁸⁰

Dibenzyl phosphite (0.5 ml, 2.3 mmol) was dissolved in dry ether (10 ml). *N*-Chlorosuccinimide (0.23 g, mmol) was added, whereupon a fine white precipitate rapidly formed. After stirring at room temperature for 2 hours under nitrogen, the reaction

mixture was filtered, to give a clear solution of the title compound in dry ether. The solvent was removed to give a clear, colourless oil which was redissolved in dry acetonitrile for use in subsequent reactions.

98 8-(*O*-dibenzylphosphoryl)-2-methylquinazolin-4-[3*H*]-one

Method 1



Prepared according to the method for compound 95, from 8-hydroxy-2-methylquinazolin-4-[3*H*]-one 37 (0.5 g, 2.8 mmol) and dibenzyl phosphorochloridate 97 in acetonitrile (1.7 g in 22.2 ml). The product (pink oil) was obtained as a white crystalline solid after recrystallisation from methanol/water (0.35 g,

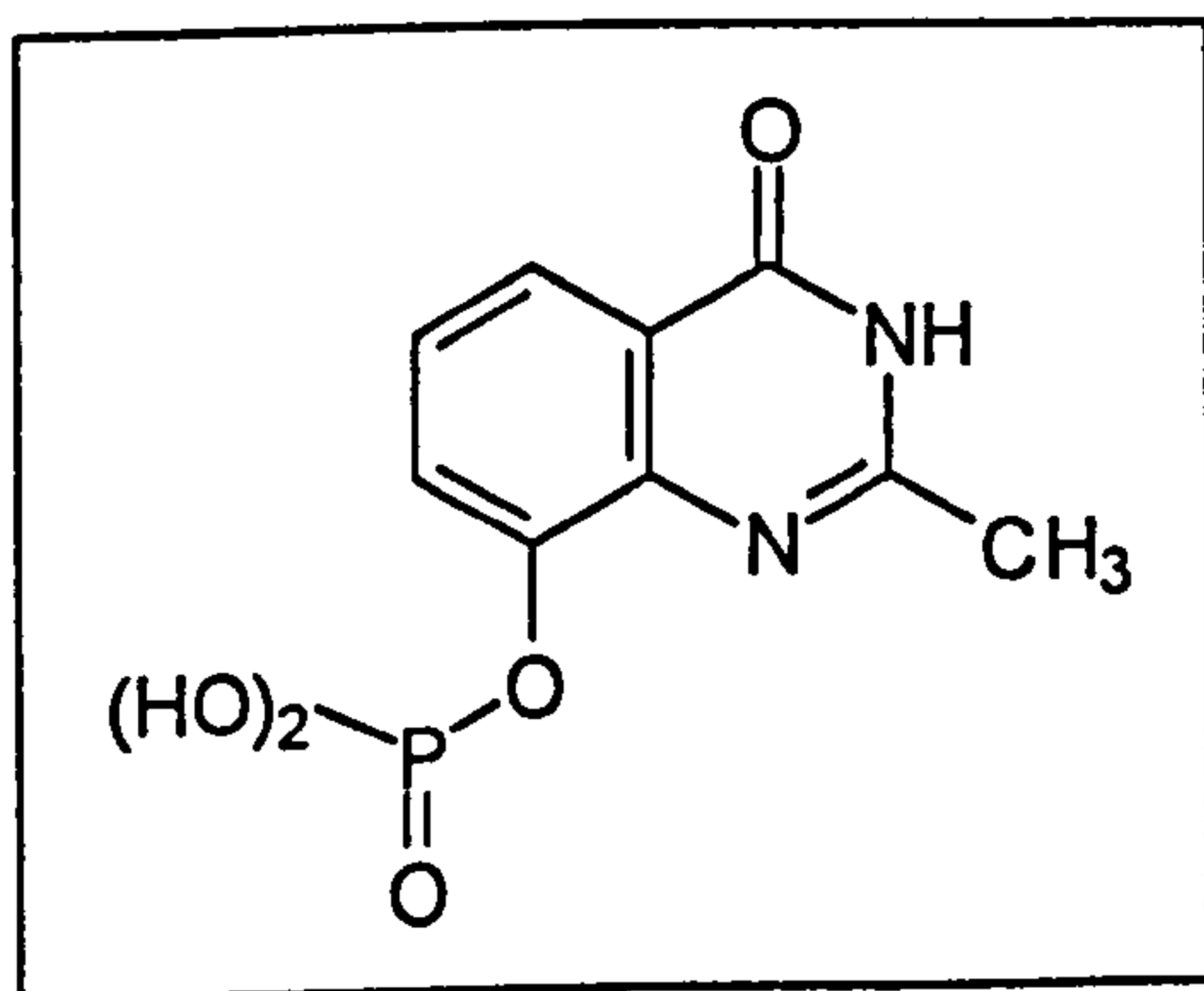
0.8 mmol, 28%), m.p. 134-135 °C.

Anal. found C 63.79, H 4.80, N 6.48, C₂₃H₂₁N₂O₃P requires C 63.30, H 4.85, N 6.42%; $\nu_{\max}/\text{cm}^{-1}$ 3168 (2° amide NH), 2803 (CH₃), 1688 (C=O), 1273 (P-O-aryl); δ_{H} (200 MHz, *d*₆-DMSO) 2.41 (s, 3H, -CH₃), 5.39 (s, 2H, CH₂-Ph), 5.44 (s, 2H, CH₂-Ph), 7.43-7.56 (m, 11H, Ar-6H, 2 × Ph-2'/3'/4'/5'/6'H), 7.67-7.73 (dd, 1H, Ar-7H), 8.01-8.05 (dd, 1H, Ar-5H), 12.45 (s, 1H, -NH); *m/z* (FAB) 437 (MH⁺, 72%), 315 ([MH-2 × OBn]⁺), 91 (Bn⁺), 77 (Ph⁺).

98 8-(*O*-dibenzylphosphoryl)-2-methylquinazolin-4-[3*H*]-oneMethod 2²⁸⁴

8-Hydroxy-2-methylquinazolin-4-[3*H*]-one **37** (0.2 g, 1.1 mmol) was placed in a three-necked flask which was fitted with septa, a thermometer and nitrogen inlet. Dry acetonitrile (25 ml) was added and the mixture was cooled to -10 °C in a salt/ice bath. Carbon tetrachloride (0.54 ml, 5.5 mmol), *N,N*-diisopropylethylamine (0.42 ml, 2.4 mmol) and *N,N*-dimethylaminopyridine (14 mg, 1.1 mmol) were added. One minute later, dropwise addition of dibenzyl phosphite (0.36 ml, 1.6 mmol) was begun (care was taken to ensure that the internal temperature of the reaction mixture did not rise above -10 °C). On completion of the reaction (as determined by TLC after 45 minutes), 0.5 M aq. potassium dihydrogen orthophosphate solution (32 ml/100 ml acetonitrile) was added and the reaction mixture was allowed to warm up to room temperature. The mixture was extracted three times with EtOAc. The combined EtOAc layers were washed with water and saturated brine solution. The organic layers were dried (Na₂SO₄), filtered and the solvent was removed under reduced pressure to give a white 'oily' solid. The product was purified by column chromatography, using DCM:MeCN (6:4) as eluent (0.24 g, 0.5 mmol, 49%), m.p. 134-135 °C.

The product was found to be identical by TLC to the product synthesised by Method 1.

96 8-(*O*-phosphoryl)-2-methylquinazolin-4-[3*H*]-one²⁸¹

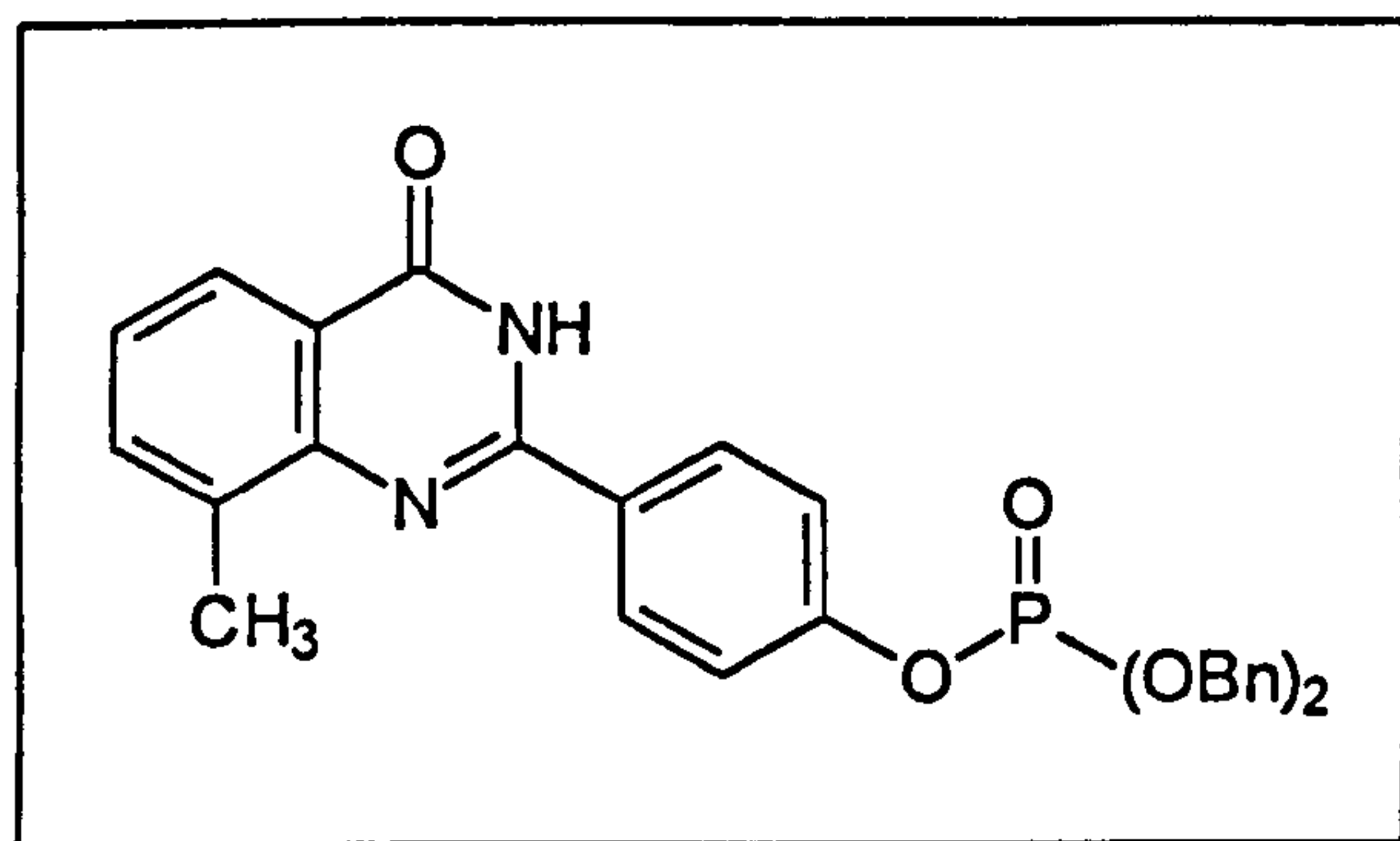
8-*O*-(dibenzylphosphoryl)-2-methylquinazolin-4-[3*H*]-one **98** (0.07 g, 0.16 mmol) was suspended in THF (5 ml, freshly distilled from sodium/benzophenone, then redistilled from LiAlH₄) and water (5 ml) with 10% palladium-carbon catalyst. The reaction vessel was placed under an atmosphere of hydrogen at ambient temperature

and pressure until no further hydrogen absorption was observed. A white precipitate formed, and a further 50 ml water was added, whereby the precipitate appeared to dissolve. The

reaction mixture was filtered through Celite to remove the catalyst. The THF and most of the water were removed under vacuum. The remaining aqueous solution was freeze-dried to give a white solid (0.03 g, 0.12 mmol, 74%), m.p. 223-224 °C.

Anal. found C 49.10, H 3.62, N 13.97, $C_{12}H_{11}N_3O_5 \cdot 0.5$ mol. HCl requires C 48.78, H 3.92, N 14.22%; $\nu_{\max}/\text{cm}^{-1}$ 3431 (br, OH), 2893 (CH_3), 1696 ($\text{C}=\text{O}$), 1244 [$\text{O}=\text{P}(\text{OH})_2$]; δ_{H} (200 MHz, d_6 -DMSO) 2.46 (s, 3H, $-\text{CH}_3$), 7.41-7.45 (t, 1H, Ar-6H), 7.82-7.86 (d, 1H, Ar-7H), 7.96 (d, 1H, Ar-5H); m/z (FAB) 257 (MH^+ , 38%).

99 8-Methyl-2-(4'-O-dibenzylphosphorylphenyl)quinazolin-4-[3H]-one



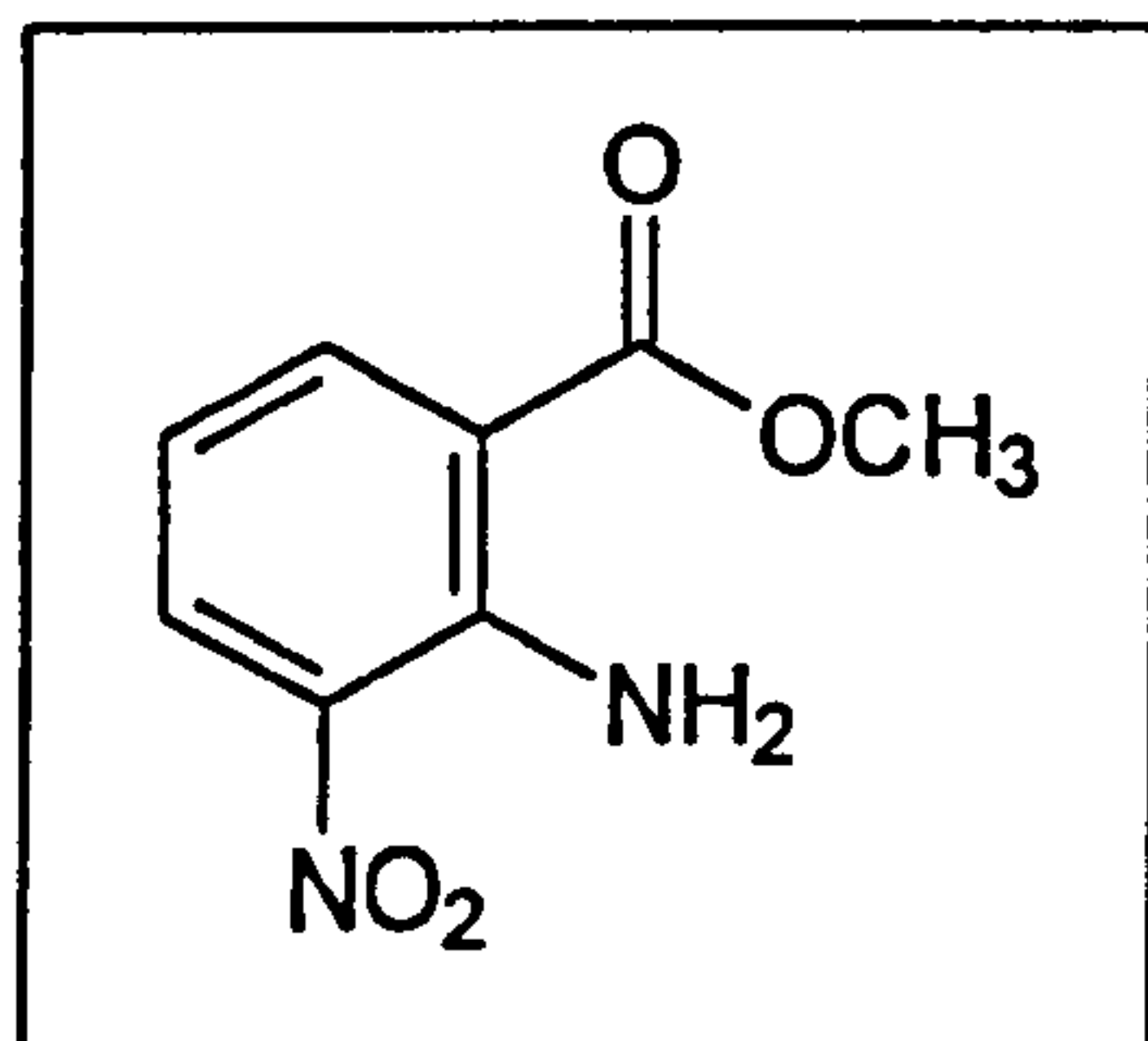
Prepared according to Method 2 for compound 98, from 8-methyl-2-(4'-hydroxyphenyl)quinazolin-4-[3H]-one 87 (1 g, 4.0 mmol). The product was purified by column chromatography using DCM:MeOH (95:5), followed by recrystallisation from

EtOAc/petrol 40/60 to give a white solid (0.53 g, 1.0 mmol, 26%), m.p. 151-153 °C.

Anal. found C 67.96, H 4.92, N 5.47, $C_{29}H_{25}N_2O_5P$ requires C 67.72, H 4.74, N 5.41%; $\nu_{\max}/\text{cm}^{-1}$ 3171 (2° amide NH), 2956 and 2922 (CH_3), 1670 ($\text{C}=\text{O}$), 1284 (P-O-aryl); δ_{H} (200 MHz, d_6 -DMSO) 2.72 (s, 3H, CH_3), 5.29 (s, 2H, $-\text{CH}_2\text{Ph}$), 5.32 (s, 2H, $-\text{CH}_2\text{Ph}$), 7.44-7.52 (m, 13 H, $2 \times \text{Ph}-2'/3'/4'/5'/6'\text{H}$, Ar-6H, Ph-2'/6'H), 7.79-7.81 (d, 1H, Ar-7H, $J = 6.9$), 8.07-8.10 (dd, 1H, Ar-5H, $J = 1.0, 7.9$), 8.33-8.36 (d, 2H, Ph-3'/5'H, $J = 8.8$), 12.65 (s, 1H, $-\text{NH}$); m/z (EI) 512 (M^+ , 1%)

8.5 Benzimidazole Experimental Procedure

104 Methyl 2-amino-3-nitrobenzoate²⁵¹



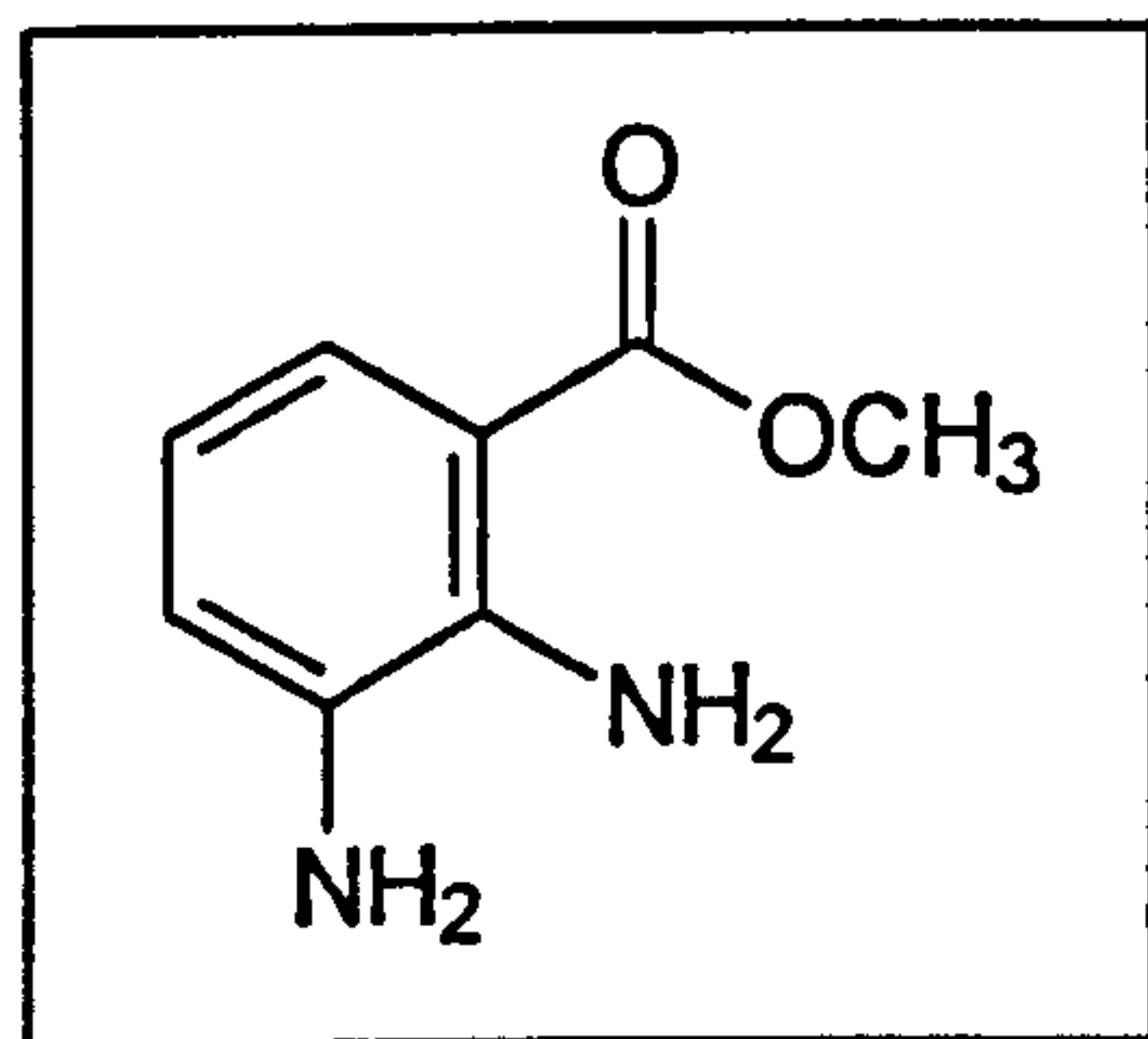
2-Amino-3-nitrobenzoic acid 103 (5.0 g, 27.5 mmol) was dissolved in methanol (80 ml) and cooled in an ice-bath. Dry hydrogen gas was passed through the solution until saturation was achieved (approx. 20 minutes), and the solution was allowed to warm to room temperature. The reaction mixture was heated at

reflux for 6 hours and then allowed to cool overnight. The resulting bright yellow precipitate was collected by filtration. The solvent was removed from the filtrate and the residue was redissolved in EtOAc and washed with water. The organic layer was dried (Na_2SO_4), filtered and the solvent was removed under reduced pressure to leave a yellow solid. Both products were found (by TLC) to contain starting material. The combined products were purified by column chromatography, using EtOAc:petrol 40/60 (1:9) as eluent to give the product as a bright yellow solid; product 3.49 g, 17.8 mmol, starting material 1.23 g, 6.75 mmol. Corrected yield : 86%, m.p. 90-92 °C (lit. m.p.²⁵¹ 95-96 °C).

Anal. found C 47.04, H 3.65, N 13.46, $\text{C}_8\text{H}_8\text{N}_2\text{O}_4 \cdot 0.25$ mol. HCl requires C 46.81, H 4.05, N 13.65%; $\nu_{\text{max}}/\text{cm}^{-1}$ 3453 (NH), 3317 (NH), 2852 (CH_3), 1515 (C=O), 1252 (NO_2); δ_{H} (200 MHz, d_6 -DMSO) 3.96 (s, 3H, OCH_3), 6.81-6.89 (t, 1H, Ar-5H, $J = 8.1$), 8.30-8.34 (dd, 1H, Ar-6H, $J = 1.6, 7.7$), 8.42-8.47 (dd, 3H, Ar-4H, -NH, $J = 1.6, 8.5$); m/z (EI) 196 (M^+ , 100%), 164 ($[\text{M}-\text{CH}_3\text{OH}]^+$).

105 Methyl 2,3-diaminobenzoate²⁵¹

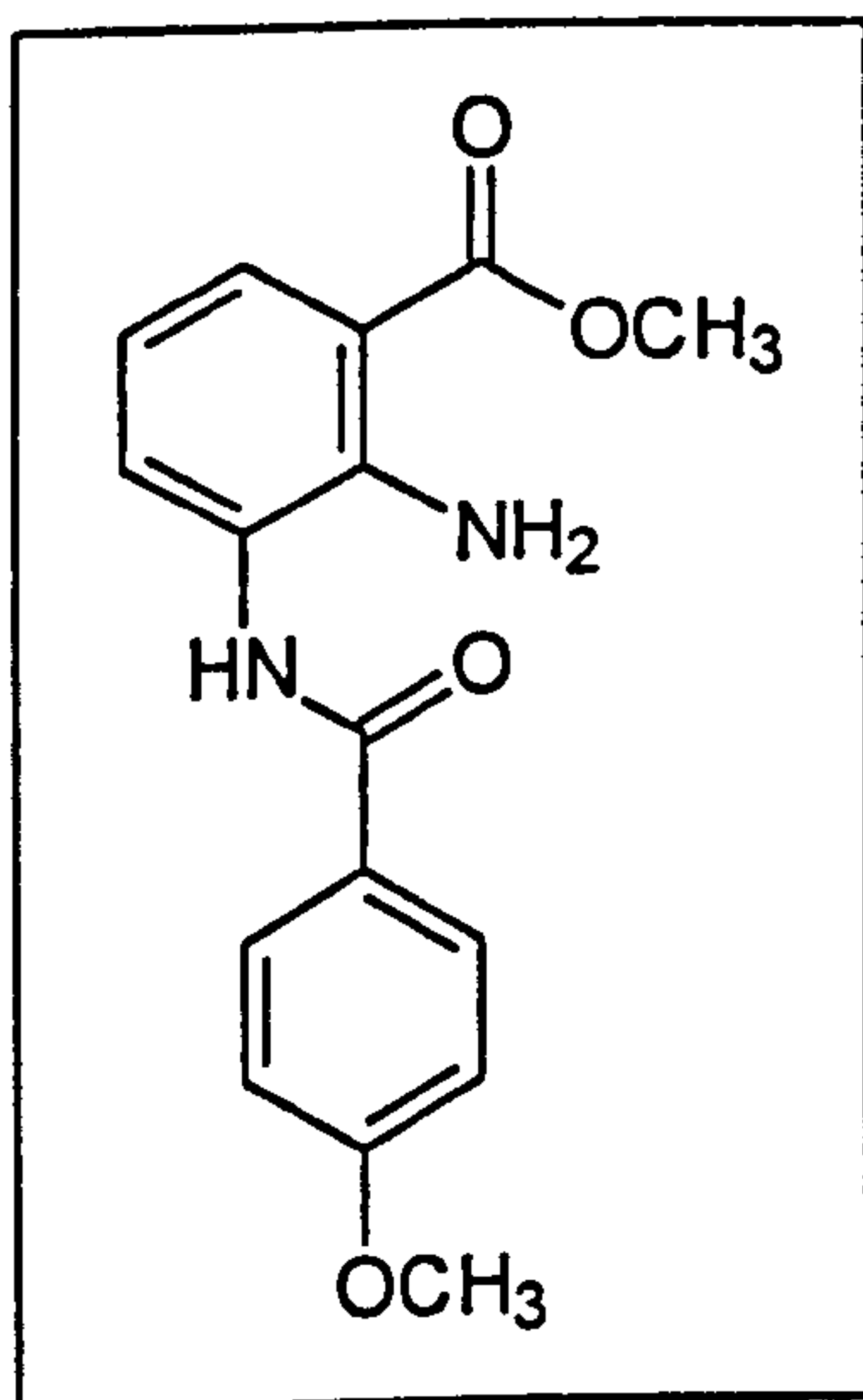
LIGHT SENSITIVE



Methyl 2-amino-3-nitrobenzoate 104 (3.5 g, 17.8 mmol) and activated 10% Pd/C catalyst (300 mg) were suspended in methanol (220 ml) and placed under an atmosphere of hydrogen, at ambient temperature and pressure, until no further hydrogen uptake was observed. The catalyst was removed by filtration

through Celite and the solvent was removed from the filtrate to leave a dark brown, 'oily' solid (3.04 g, >100% crude). The product was used for subsequent reactions without further purification.

δ_{H} (200 MHz, d_6 -DMSO) 3.48 (br s, 2H, NH_2), 3.85 (s, 3H, CO_2CH_3), 4.22 (br s, 2H, NH_2), 6.44-6.52 (t, 1H, Ar-5H, $J = 7.8$), 6.78-6.82 (dd, 1H, Ar-4H, $J = 1.5, 7.5$), 7.17-7.21 (dd, 1H, Ar-6H, $J = 1.5, 8.1$).

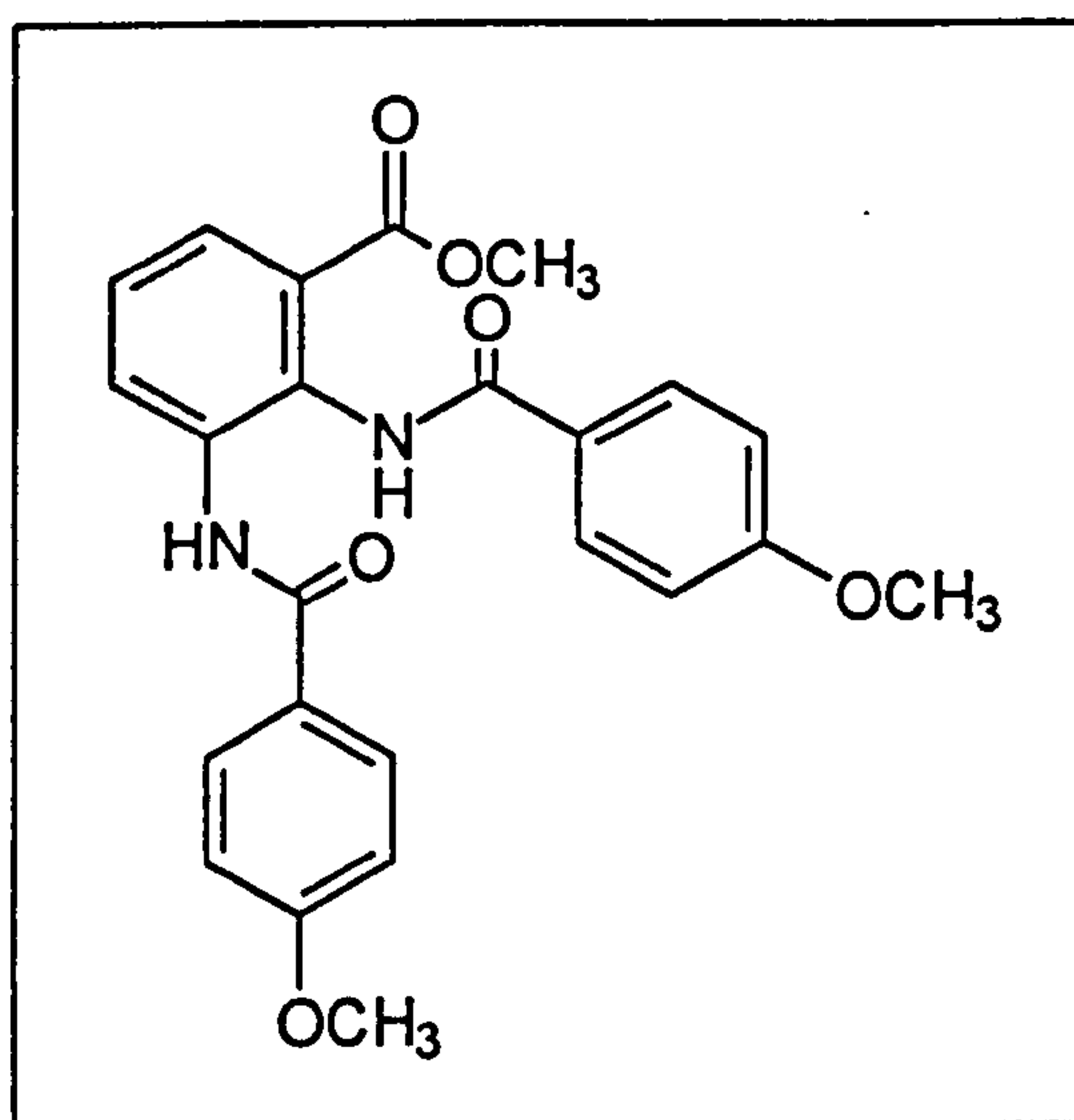
117 Methyl 2-amino-3-*N*-(4'-methoxybenzoyl)aminobenzoate²⁵¹

Methyl 2,3-diaminobenzoate hydrochloride salt 119 (1.36 g, 6.7 mmol) was added to a solution of dry THF (50 ml), containing dry triethylamine (2.8 ml, 20.1 mmol) and 4-dimethylaminopyridine (41 mg, 5 ol. %). The reaction mixture was cooled in a salt/ice bath. 4-Methoxybenzoyl chloride (1.0 ml, 6.7 mmol) was dissolved in dry THF (50 ml) and added dropwise to the reaction mixture over a period of 30 minutes. The reaction mixture was allowed to warm to room temperature and stirred for 48 hours. The solvent was removed under vacuum and the crude residue was purified using

column chromatography, using DCM:MeCN (95:5) as eluent to give a pale brown solid (0.51 g, 1.7 mmol, 25%), m.p. 176-179 °C (lit. m.p.²⁵¹ 179-180 °C)

δ_{H} (200 MHz, d_6 -DMSO) 3.93 (s, 3H, Ar-OCH₃), 3.94 (s, 3H, CO₂CH₃), 6.61 (br s, 2H, NH₂), 6.68-6.76 (t, 1H, Ar-5H, $J = 7.9$), 7.12-7.18 (dd, 2H, Ph-3''/5H, $J = 8.9$), 7.43-7.47 (dd, 1H, Ar-4H, $J = 1.5, 7.6$), 7.79-7.84 (dd, 1H, Ar-6H, $J = 1.6, 8.1$), 8.06-8.12 (dd, 2H, Ph-2'/6'H, $J = 8.8$), 9.7 (br s, 1H, NH).

118 Methyl-2,3-*N,N*-bis-(4'-methoxybenzoyl)aminobenzoate

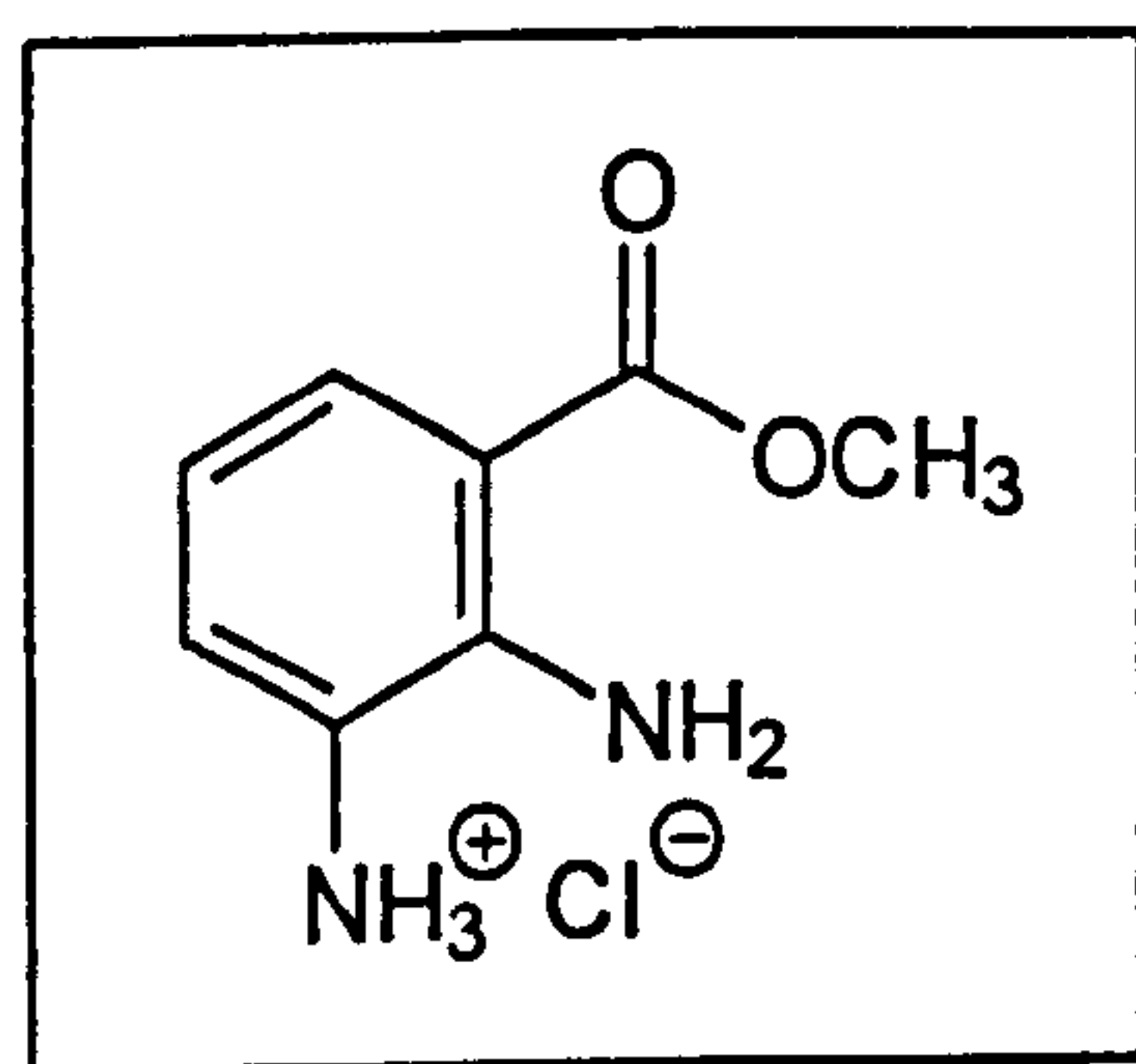


This compound was isolated during the synthesis of 117, methyl 2-amino-3-*N*-(4'-methoxybenzoyl)aminobenzoate, as a pale brown solid (0.55 g, 1.3 mmol, 19%), m.p. 151-152 °C.

Anal. found C 65.22, H 5.06, N 6.35 C₂₄H₂₂N₂O₆·0.3 mol. H₂O requires C 65.53, H 5.18, N 6.37%; $\nu_{\text{max}}/\text{cm}^{-1}$ 3327 and 3207 (1° amide NH), 2993, 2951 and 2840 (CH₃), 1670 and 1657 (C=O); δ_{H} (200 MHz, d_6 -DMSO) 3.82 (s, 3H, CO₂CH₃), 3.92 (s, 3H, 4'-OCH₃),

3.93 (s, 3H, 4'-OCH₃), 7.15-7.19 (m, 4H, 2 × Ph-3'/5'H), 7.51-7.80 (t, 1H, Ar-5H), 7.77-7.80 (dd, 1H, Ar-4H), 7.99-8.04 (m, 5H, Ar-6H, 2 × Ph-2'/6'H); m/z (EI) 434 (M⁺, 44.4%), 402 ([M-CH₃OH]⁺), (Ph⁺).

119 Methyl 2,3-diaminobenzoate hydrochloride salt



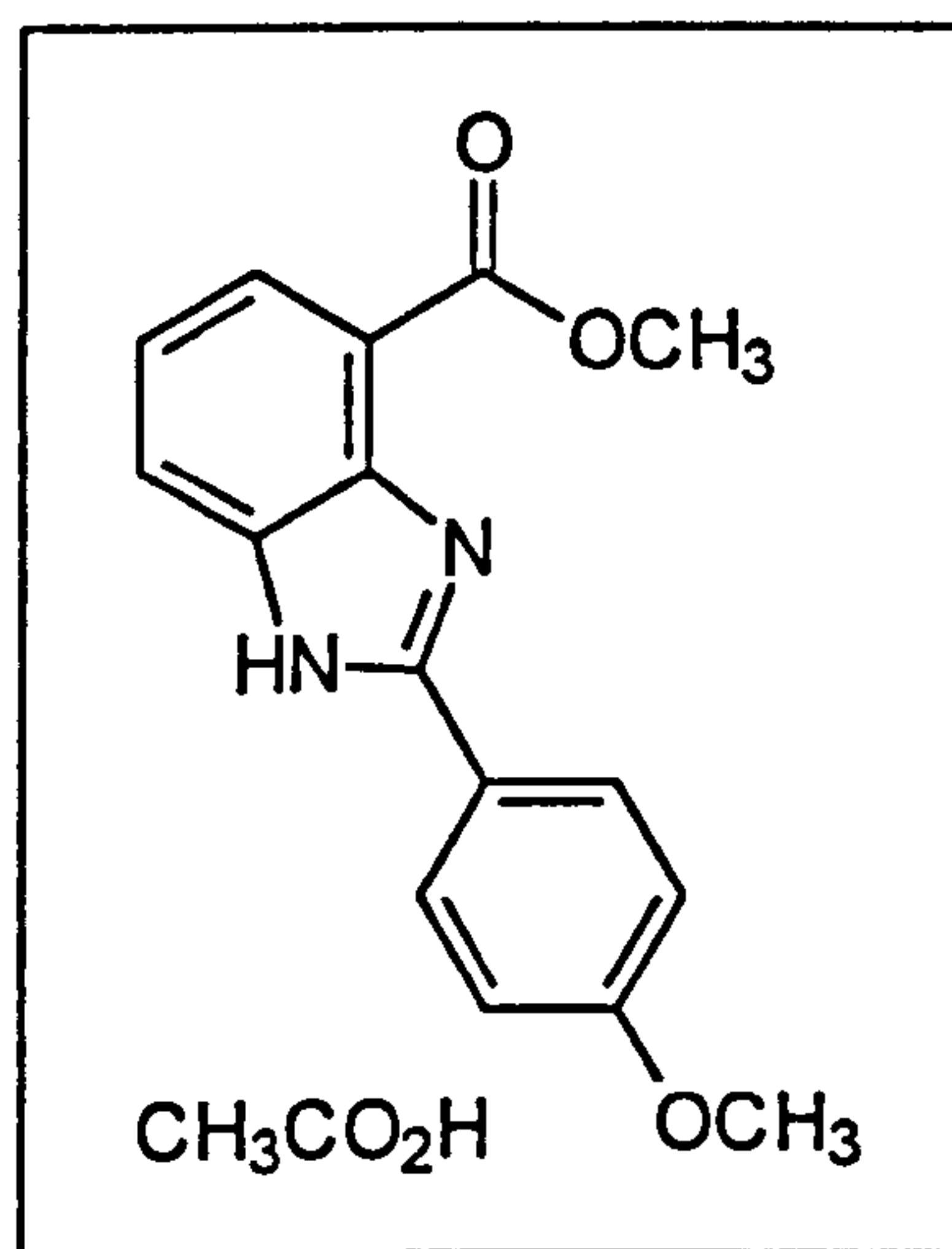
Methyl 2-amino-3-nitrobenzoate 104 (1.5 g, 7.6 mmol) was dissolved in dry methanol (60 ml) and the solution was cooled in a salt/ice bath. Acetyl chloride (2.7 ml, 38.2 mmol) and activated 10% Pd/C catalyst (250 mg) were added to the cooled solution. The reaction vessel was placed under an atmosphere of hydrogen, at ambient temperature and pressure, until no further hydrogen

uptake was observed. The catalyst was removed by filtration through Celite and the solvent

was removed from the filtrate to give a pink solid (1.36 g, 6.7 mmol, 88%). The product was used for subsequent reactions without further purification.

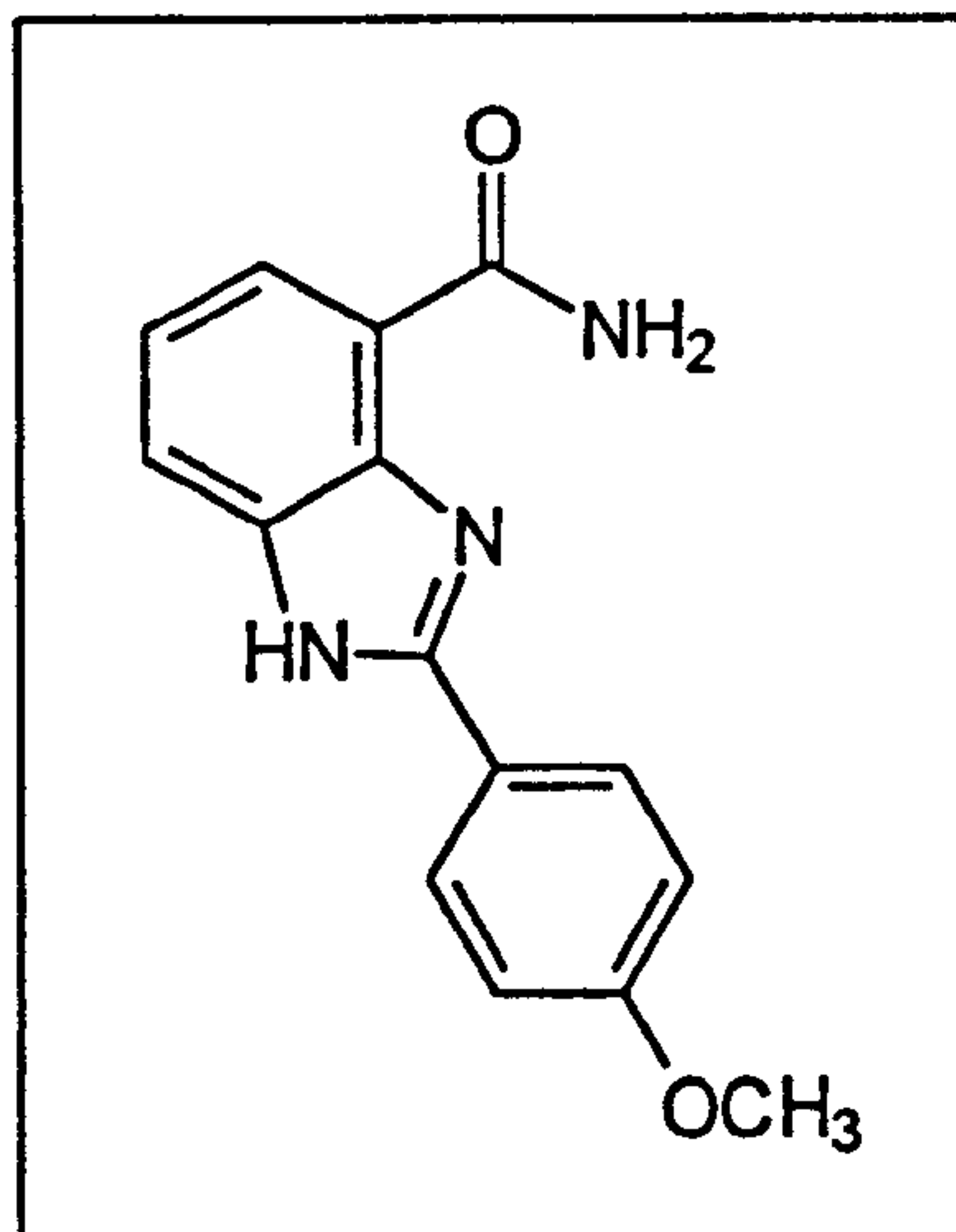
δ_{H} (200 MHz, d_6 -DMSO) 3.94 (s, 3H,), 6.77-6.85 (t, 1H, Ar-5H, $J = 7.9$), 7.63-7.67 (dd, 1H, Ar-4H, $J = 1.5, 7.7$), 7.89-7.93 (dd, 1H, Ar-6H, $J = 1.5, 8.1$).

120 Methyl 2-(4'-methoxyphenyl)-1H-benzimidazole-4-carboxylate acetate salt²⁵¹



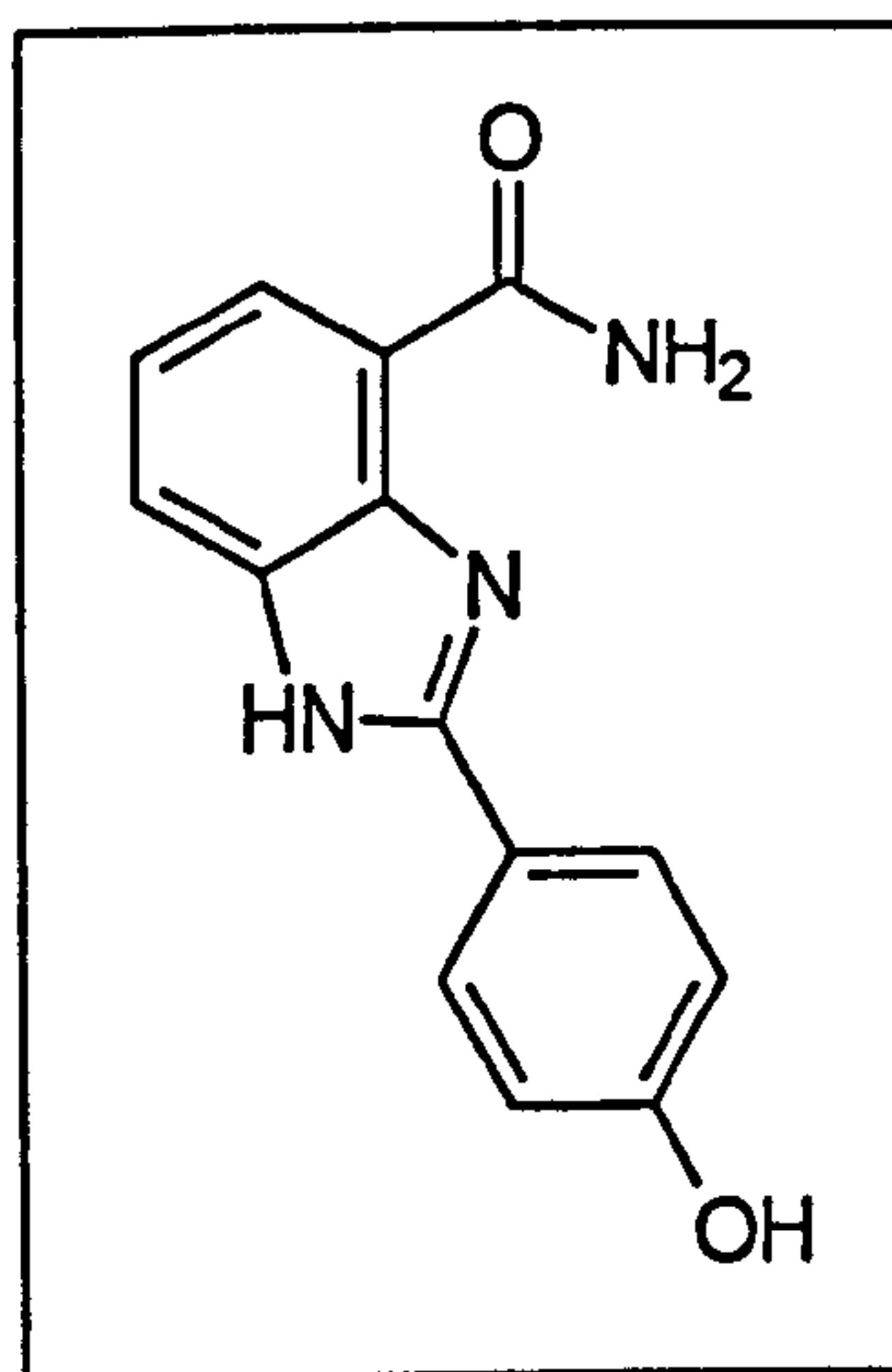
Methyl 2-amino-3-*N*-(4'-methoxybenzoyl)aminobenzoate **117** (1.55 g, 5.2 mmol) was added to glacial acetic acid (40 ml), immersed in a preheated oil bath (120 °C), and the mixture was heated for 1 hour. The reaction mixture was allowed to cool to room temperature and the solvent was removed under reduced pressure. The residue was purified by column chromatography, using DCM:MeCN (95:5) as eluent (1.3 g, 3.8 mmol, 74%), m.p. 140-142 °C (lit. m.p.²⁵¹ 141-142 °C).

δ_{H} (200 MHz, d_6 -DMSO) 2.02 (s, 3H, CH₃CO₂H), 3.96 (s, 3H, CO₂CH₃), 4.09 (2, 3H, Ph-4'-OCH₃), 7.20-7.24 (d, 2H, Ph-3'/5'H, $J = 8.6$), 7.38-7.46 (t, 1H, Ar-5H, $J = 7.8$), 7.90-7.94 (d, 1H, Ar-4H, $J = 7.8$), 8.02-8.06 (d, 1H, Ar-6H, $J = 7.6$), 8.36-8.41 (d, 2H, Ph-2'/6'H, $J = 8.6$), 12.8 (br 2, 1H, -NH).

121 2-(4'-Methoxyphenyl)-1*H*-benzimidazole-4-carboxamide²⁵¹

Methyl 2-(4'-methoxyphenyl)-1*H*-benzimidazole-4-carboxylate acetate salt **120** (1.3 g, 3.8 mmol) was added to freshly condensed liquid ammonia (80 ml). The reaction mixture was heated to 80 °C within a sealed vessel, generating a pressure of 40 atm, for 24 hours. The ammonia was evaporated and the crude residue was washed with water (10 ml) and collected as pale brown solid after filtration (1.23 g, 4.6 mmol, 87%), m.p. 263-265 °C (lit. m.p.²⁵¹ 261-263 °C).

$\nu_{\max}/\text{cm}^{-1}$ 3322 (br, NH), 2840 (OCH₃), 1656 and 1608 (C=O); δ_{H} (200 MHz, *d*₆-DMSO) 3.95 (s, 3H, OCH₃), 7.22-7.26 (d, 2H, Ph-3'/5'H, *J* = 8.9), 7.36-7.44 (t, 1H, Ar-5H, *J* = 7.8), 7.78-7.82 (d, 1H, Ar-4H, *J* = 7.1), 7.87 (br s, 1H, -NH), 7.91-7.95 (d, 1H, Ar-6H, *J* = 7.0), 8.27-8.31 (d, 2H, Ph-2'/6'H, *J* = 8.8), 9.5 (br s, 1H, -NH), 12.7 (br s, 1H, -NH); *m/z* (EI) 267 (M⁺, 44%), 250 ([M-NH₃]⁺), 236 ([M-OCH₃]⁺).

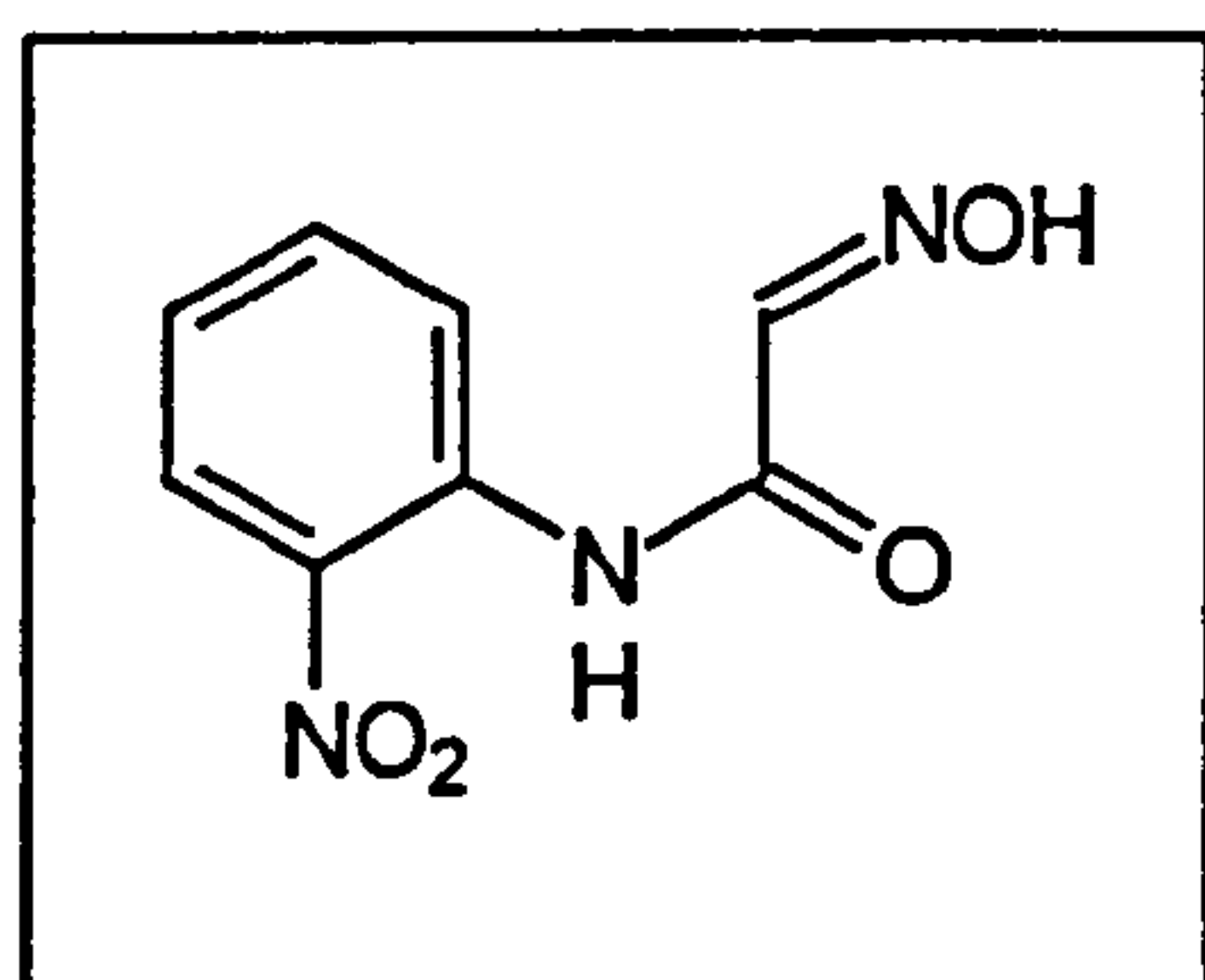
109 2-(4'-Hydroxyphenyl)-1*H*-benzimidazole-4-carboxamideMethod 1²⁵¹

Prepared according to Standard Procedure D, from 2-(4'-methoxyphenyl)-1*H*-benzimidazole-4-carboxamide **121** (1.2 g, 4.5 mmol). The product was purified by column chromatography, using DCM:MeCN (6:4) as eluent, and recrystallised from methanol/water to give a cream solid (0.49 g, 2.0 mmol, 44%), m.p. 267-268 °C (lit. m.p.²⁵¹ 266-267 °C).

Anal. found C 64.19, H 4.43, N 16.07, C₁₄H₁₁N₃O₂·0.5 mol. H₂O requires C 64.11, H 4.61, N 16.03%; $\nu_{\max}/\text{cm}^{-1}$ 3421 and 3309 (1° amide NH), 3382 (OH), 3151 (NH), 1649 (C=N), 1618 and 1577 (C=O); δ_{H} (200 MHz, *d*₆-DMSO) 7.03-7.07 (d, 2H, Ph-3'/5'H, *J* = 8.7), 7.34-7.42 (t, 1H, Ar-5H, *J* = 7.8), 7.75-7.79 (d,

1H, Ar-4H, $J = 7.9$), 7.87 (br s, 1H, -NH), 7.91-7.92 (d, 1H, Ar-6H, $J = 7.2$), 8.15-8.20 (d, 2H, Ph-2'/6'H, $J = 8.6$); m/z (EI) 253 (M^+ , 100%), 236 ($[M-NH_3]^+$), 208 ($[M-CONH_3]^+$).

126 Isonitrosoacet-*o*-nitroanilide³⁰¹

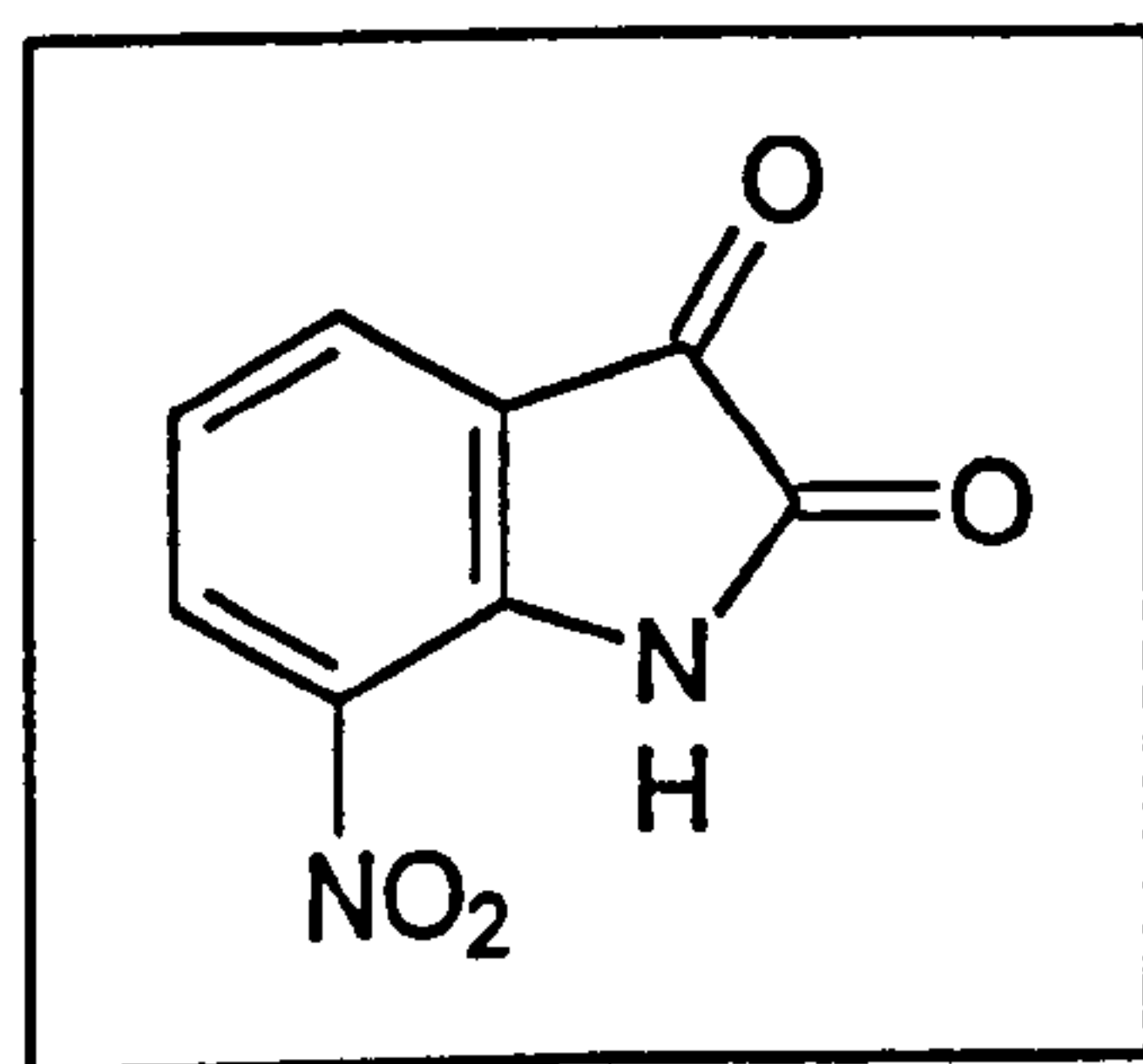


A mixture of water (10 ml), chloral hydrate (0.54 g) and anhydrous sodium sulfate (3.75 g) was heated to 40 °C to obtain complete dissolution. A solution of *o*-nitroaniline **125** (0.45 g, 3.3 mmol) and concentrated HCl (0.85 ml) in water (10 ml) was added and the reaction mixture was vigorously stirred. A solution

of hydroxylamine hydrochloride (0.71 g) in water (2.5 ml) was added and the reaction mixture was heated at reflux for 15 minutes. The solution was allowed to cool to room temperature and the resulting bright yellow precipitate was collected by filtration and washed with water (0.22 g, 1.0 mmol, 32%), m.p. 143-145 °C (lit. m.p.³⁰¹ 145.5-146 °C).

Anal. found C 45.53, H 2.94, N 19.77, $C_8H_7N_3O_4 \cdot 0.1$ mol. HCl requires C 45.15, H 3.36, N 19.75%; $\nu_{\max}/\text{cm}^{-1}$ 3259 (br, NH), 1671 (C=O), 1342 (NO_2); δ_{H} (200 MHz, d_6 -DMSO) 7.42-7.51 (m, 1H, Ar-5H), 7.73 (s, 1H, CHNOH), 7.84-7.92 (m, 1H, Ar-4H), 8.22-8.27 (dd, 1H, Ar-6H, $J = 1.5, 8.4$), 8.38-8.42 (dd, 1H, Ar-3H, $J = 1.3, 8.4$), 11.1 (s, 1H, -OH), 12.8 (s, 1H, -NH); m/z (EI) 209 (M^+ , 53%), 160 ($[M-\text{NO}_2]^+$).

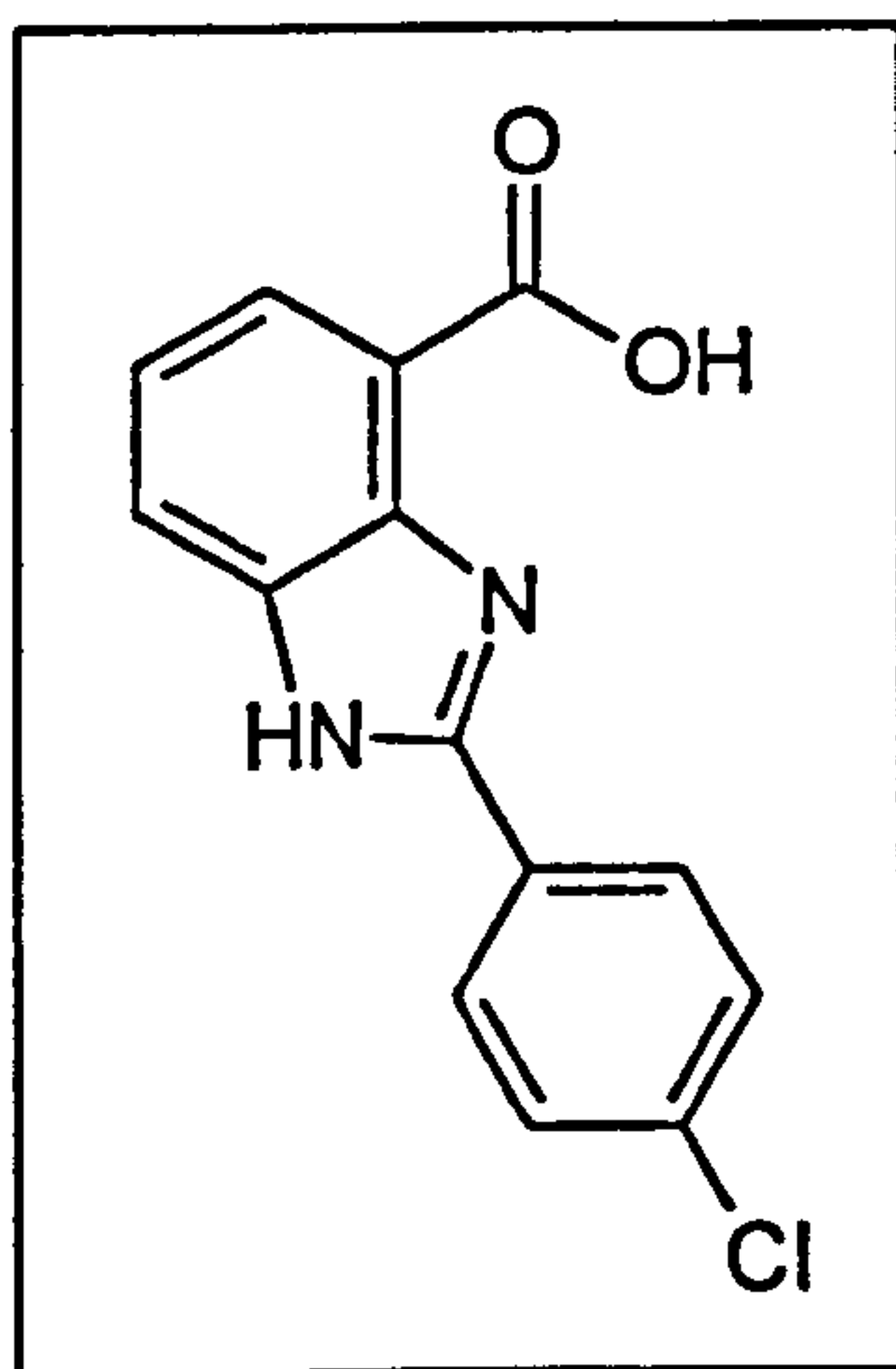
127 Attempted Preparation of 7-nitroisatin³⁰¹



Concentrated sulfuric acid (2 ml) was cautiously heated to 70 °C. Isonitrosoacet-*o*-nitroanilide **126** (0.15 g, 0.7 mmol) was added in small portions (to ensure the internal temperature did not rise more than a few degrees). The temperature was maintained at 70 °C (15 min). The reaction mixture was carefully added to crushed ice and left to stand (30 min). An attempt to filter off any product proved unsuccessful, as did

extraction of the crude yellow filtrate. No positive analytical results were obtained for this compound.

133 2-(4'-Chlorophenyl)-1H-benzimidazole-4-carboxylate³⁰³



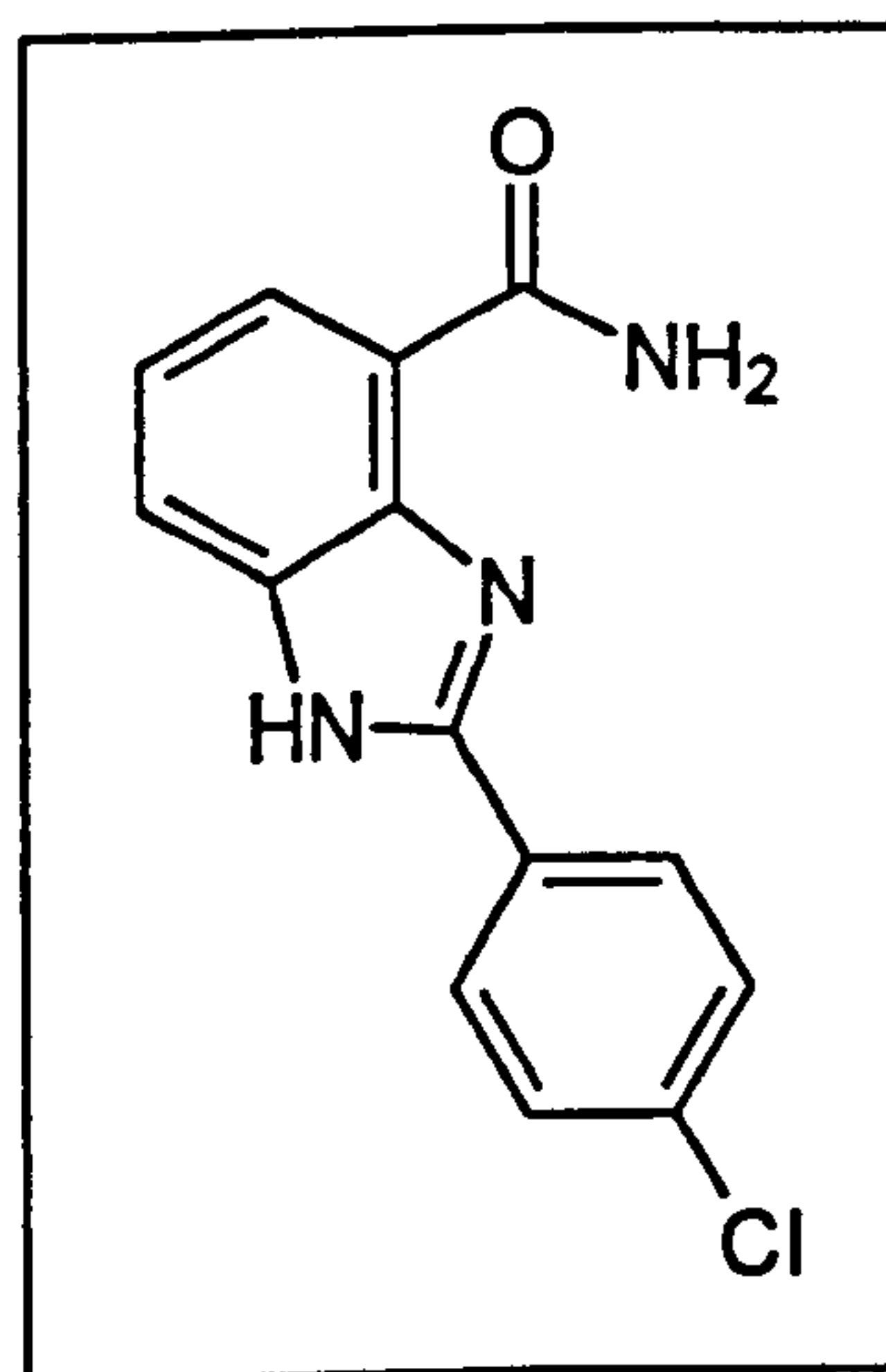
Prepared according to Standard Procedure E, from 3-nitroanthranilic acid 103 (1.0 g, 5.5 mmol) and 4-chlorobenzaldehyde (1.08 g, 7.7 mmol) to give the product as a pale brown solid.

Yield : 1.2 g, 4.4 mmol, 80%, m.p. 306–308°C (lit. m.p.³⁰³ >300 °C).

Anal. found C 61.56, H 3.14, N 10.21, C₁₄H₉N₂O₂Cl requires C 61.66, H 3.33, N 10.27%; $\nu_{\max}/\text{cm}^{-1}$ 3395 (br, OH), 3092 (NH), 1715 (CO₂H), 755 (C-Cl); δ_{H} (200 MHz, *d*₆-DMSO) 7.53–7.60 (t, 1H, Ar-

5H, *J* = 7.4), 7.77–7.81 (d, 2H, Ph-2'/6'H, *J* = 7.7), 8.01–8.05 (d, 1H, Ar-4H, *J* = 7.5), 8.09–8.13 (d, 1H, Ar-6H, *J* = 7.8), 8.44–8.48 (d, 2H, Ph-3'/5'H, *J* = 7.9); *m/z* (EI) 272 (M⁺ [³⁵Cl]), 6 %), 274 (M⁺ [³⁷Cl]), 254, 256 ([M-H₂O]⁺), 226, 228 ([M-CH₂O₂]⁺).

134 2-(4'-Chlorophenyl)-1H-benzimidazole-4-carboxamide

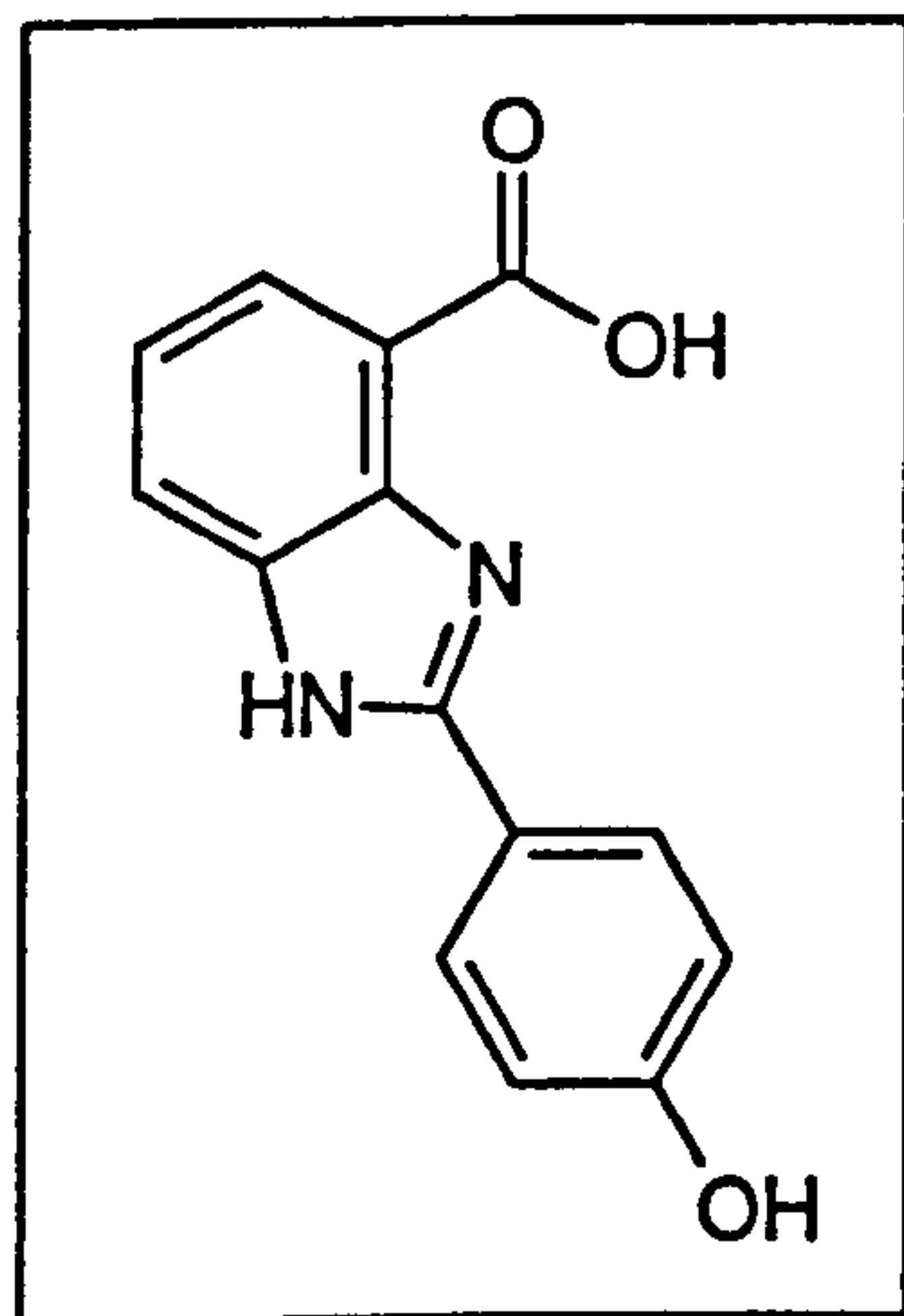


Prepared according to Standard Procedure F, from 2-(4'-chlorophenyl)-1H-benzimidazole-4-carboxylate 133 (0.5 g, 1.8 mmol). The product was recrystallised from methanol to give pale brown crystals (0.23 g, 0.9 mmol, 47%), m.p. 262–264 °C.

Anal. found C 61.60, H 3.81, N 15.29, C₁₄H₁₀N₃OCl.0.1 mol. CH₃OH requires C 61.88, H 3.71, N 15.47%; $\nu_{\max}/\text{cm}^{-1}$ 3389 (NH), 3173 (NH), 1657 and 1596 (C=O), 758 (C-Cl); δ_{H} (200 MHz, *d*₆-DMSO) 7.43–7.50 (t, 1H, Ar-5H, *J* = 7.8), 7.76–7.80 (d, 2H, Ph-

3'/5'H, $J = 8.4$), 7.84-7.88 (d, 1H, Ar-4H, $J = 7.8$), 7.93 (br s, 1H, -NH), 7.96-8.00 (d, 1H, Ar-4H, $J = 7.5$), 8.35-8.40 (d, 2H, Ph-2'/6'H, $J = 8.5$), 9.6 (br s, 1H, -NH), 13.6 (br s, 1H, -NH); m/z (EI) 271 (M^+ [^{35}Cl], 100%), 273 (M^+ [^{37}Cl]), 226, 228 ($[M-\text{CONH}_3]^+$).

135 2-(4'-Hydroxyphenyl)-1H-benzimidazole-4-carboxylate³⁰³



Prepared according to Standard Procedure E, from 3-nitroanthranilic acid 103 (5.0 g, 27 mmol) and 4-hydroxybenzaldehyde (4.7 g, 38 mmol) to give the product as a brown solid.

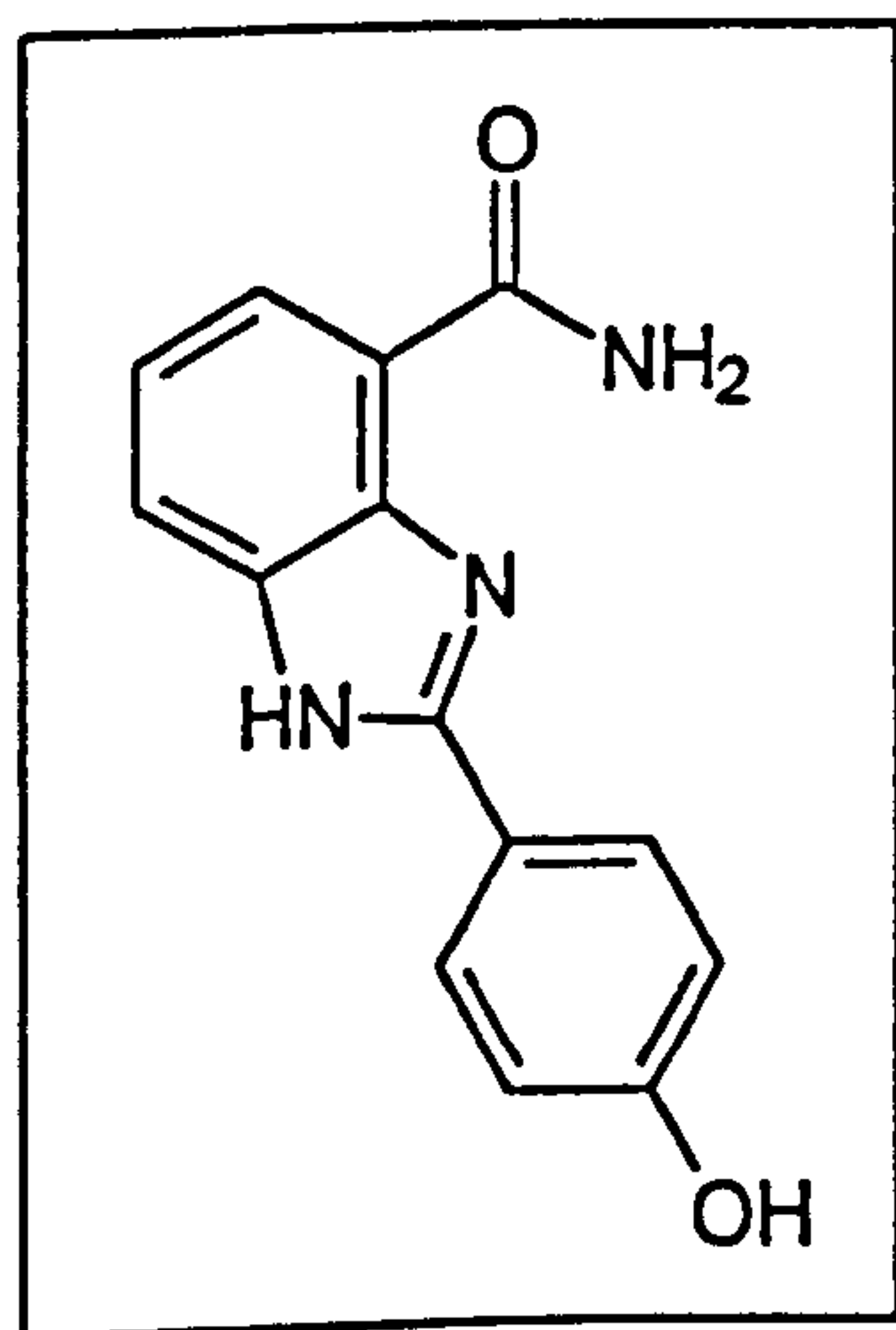
Yield : 1.2 g, 4.7 mmol, 17%.

Anal. found C 57.48, H 4.02, N 9.47, $\text{C}_{14}\text{H}_{10}\text{N}_2\text{O}_3 \cdot 1.0$ mol. HCl requires C 57.84, H 3.81, N 9.64%; $\nu_{\text{max}}/\text{cm}^{-1}$ 3410 (br, NH), 3170 (br, OH), 1613 (C=O), 1595 (CO_2H); δ_{H} (200 MHz, d_6 -DMSO)

7.01-7.06 (d, 2H, Ph-2'/6'H, $J = 8.3$), 7.24-7.31 (t, 1H, Ar-5H, $J = 7.7$), 7.76-7.80 (d, 2H, Ar-4/6H, $J = 7.7$), 8.15-8.19 (d, 2H, Ph-3'/5'H, $J = 8.5$); m/z (EI) 254 (M^+ , 91%), 236 ($[M-\text{H}_2\text{O}]^+$), 208 ($[M-\text{CH}_2\text{O}_2]^+$).

109 2-(4'-Hydroxyphenyl)-1H-benzimidazole-4-carboxamide

Method 2



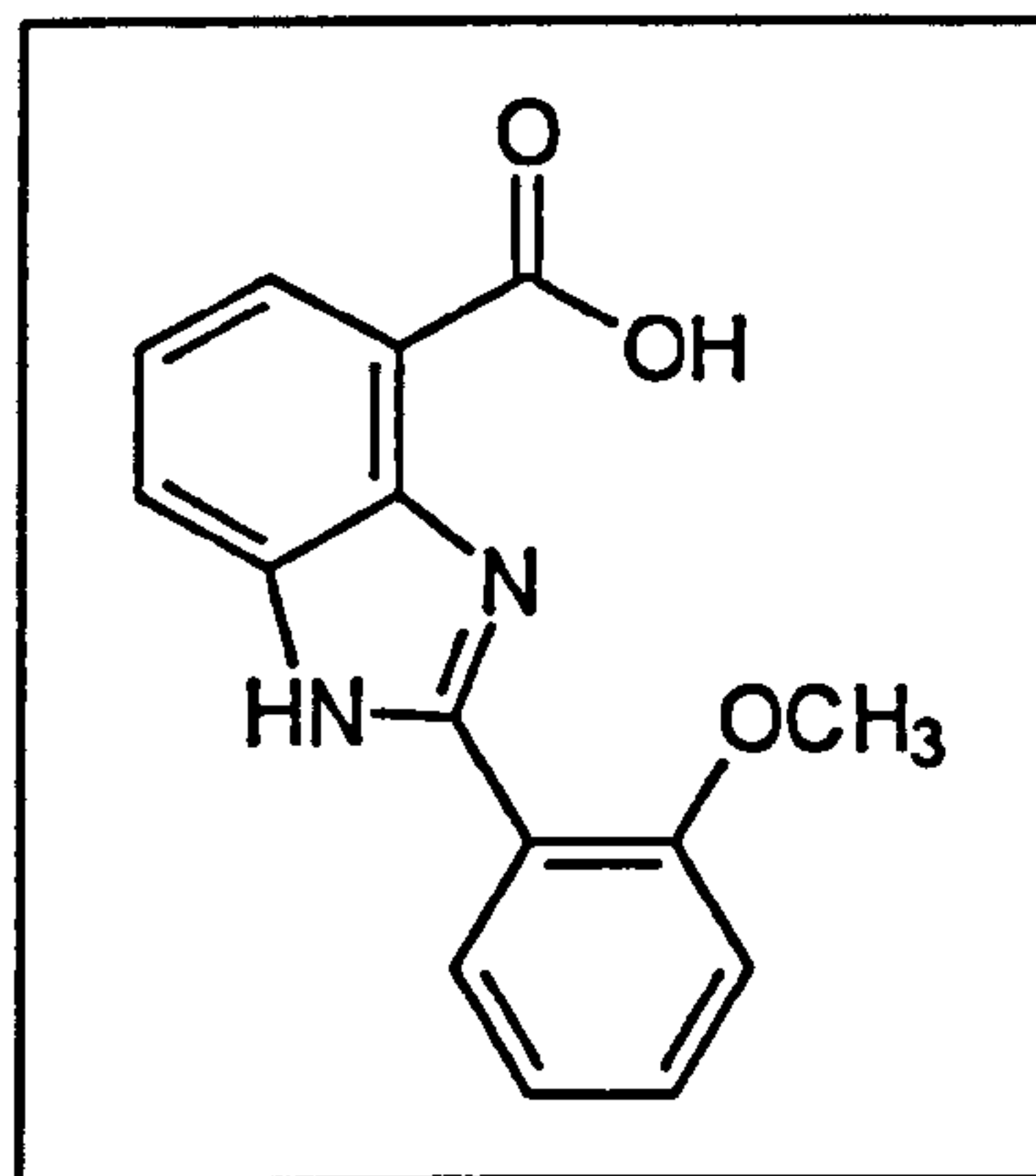
Prepared according to Standard Procedure F, from 2-(4'-hydroxyphenyl)-1H-benzimidazole-4-carboxylate 134 (1.0 g, 3.9 mmol). The cream compound was found to be identical to the one synthesised by Method 1.

Yield : 0.74 g, 2.9 mmol, 75%), m.p. 266-267 °C, (lit. m.p.²⁵¹ 266-267 °C).

δ_{H} (200 MHz, d_6 -DMSO) 7.03-7.07 (d, 2H, Ph-3'/5'H, $J = 8.7$), 7.34-7.42 (t, 1H, Ar-5H, $J = 7.8$), 7.75-7.79 (d, 1H, Ar-4H, $J =$

7.0), 7.79 (br s, 1H, -NH), 7.91-7.94 (d, 1H, Ar-6H, $J = 6.8$), 8.15-8.19 (d, 2H, Ph-2'/6'H, $J = 8.7$).

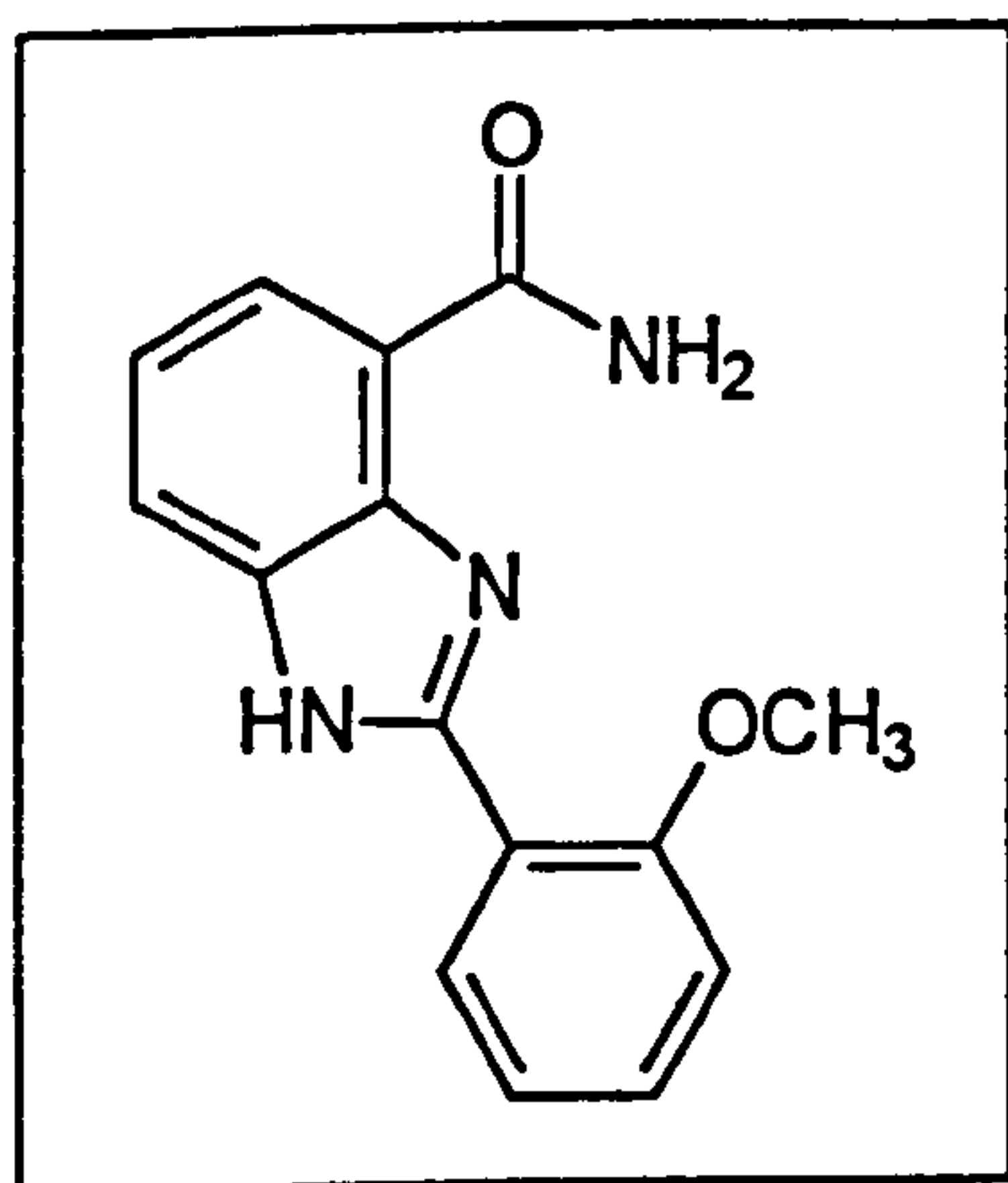
136 2-(2'-Methoxyphenyl)-1H-benzimidazole-4-carboxylate³⁰³



Prepared according to Standard Procedure E, from 3-nitroanthranilic acid 103 (0.75 g, 4.1 mmol) and 2-methoxybenzaldehyde (0.79 g, 5.7 mmol). The product was purified by column chromatography, using DCM:MeOH (9:1) as eluent, followed by recrystallisation from methanol to give a cream crystalline solid (0.56 g, 2.1 mmol, 50%), m.p. 278-279 °C (lit. m.p.³⁰³ 278-279. °C).

Anal. found C 67.53, H 4.49, N 10.40, $C_{15}H_{12}N_2O_3$ requires C 67.16, H 4.51, N 10.44%; $\nu_{\max}/\text{cm}^{-1}$ 3419 (NH), 2838 (OCH_3), 1677 (CO_2H); δ_{H} (200 MHz, d_6 -DMSO) 4.19 (s, 3H, - OCH_3), 7.30-7.33 (t, 1H, Ar-5H), 7.42-7.50 (m, 2H, Ar-4H, Ph-4'H), 7.63-7.66 (m, 1H, Ph-5'H), 7.91-7.95 (d, 1H, Ph-6'H), 8.05-8.09 (d, 1H, Ph-3'H), 8.48-8.52 (d, 1H, Ar-6H), 11.9 (br 2, 1H, -OH), 13.6 (br s, 1H, -NH) m/z (EI) 268 (M^+ , 97%), 222 ($[\text{M}-\text{CH}_2\text{O}_2]^+$).

137 2-(2'-Methoxyphenyl)-1H-benzimidazole-4-carboxamide

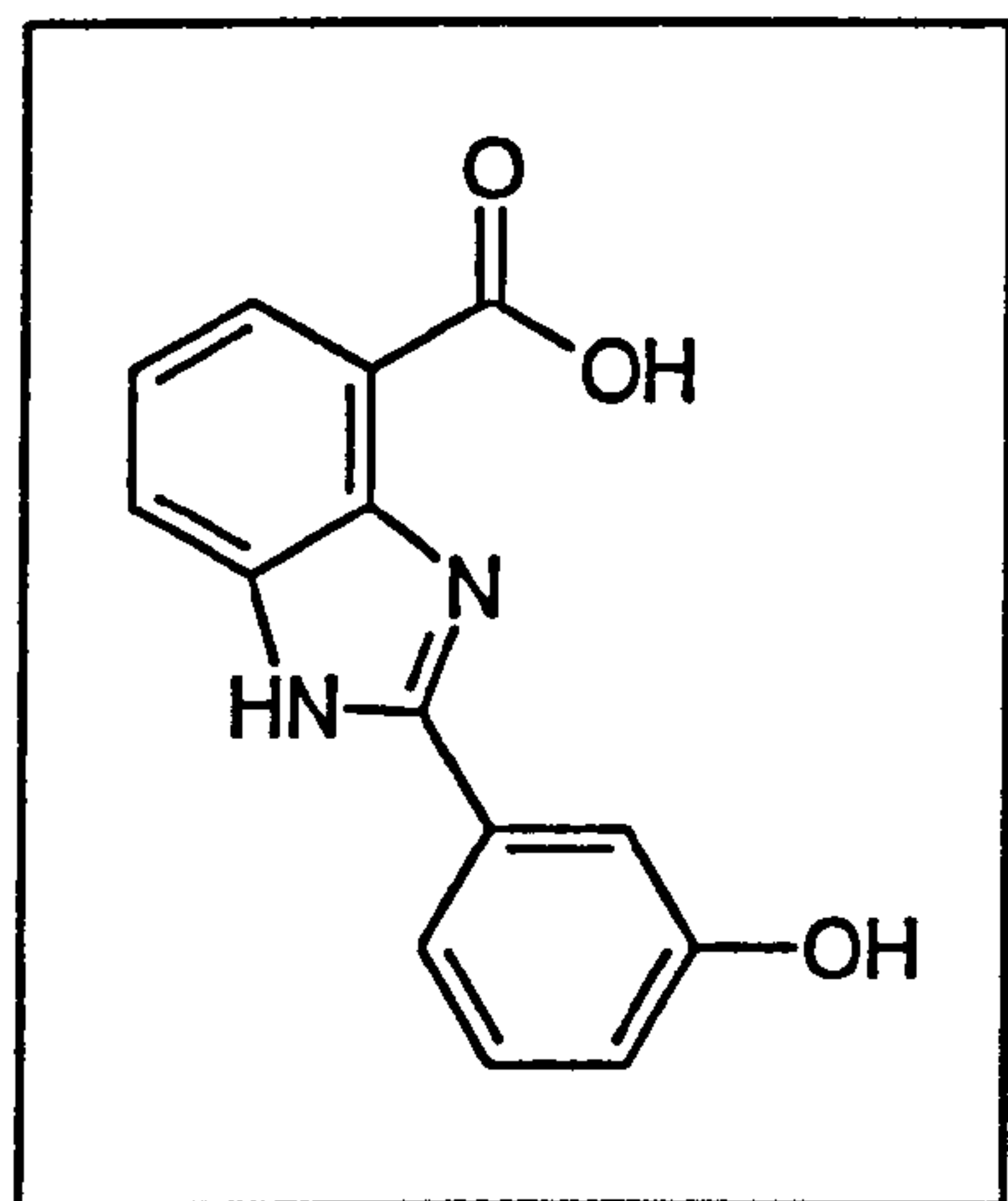


Prepared according to Standard Procedure F, from 2-(2'-methoxyphenyl)-1H-benzimidazole-4-carboxylate 136 (0.12 g, 0.4 mmol). The product was obtained as a white crystalline solid after recrystallisation from methanol/water (0.09 g, 0.3 mmol, 75%), m.p. 224-226 °C.

Anal. found C 67.15, H 4.80, N 15.45, $C_{15}H_{13}N_3O_2$ requires C 67.40, H 4.90, N 15.72%; $\nu_{\max}/\text{cm}^{-1}$ 3421 (NH), 3321 and 3154 (br, 1° amide NH), 2849 (OCH_3), 1673 (C=O); δ_{H} (200 MHz, d_6 -DMSO) 4.15 (s, 3H, OCH_3), 7.23-7.31 (t, 1H, Ar-5H, $J = 7.5$), 7.36-7.47 (m, 2H, Ph-4'H and NH), 7.61-7.69 (t, 1H, Ph-

5'H, $J = 7.9$), 7.89-7.97 (m, 3H, Ar-4H, Ph-3'/6'H), 8.47-8.51 (d, 1H, Ar-6H, $J = 7.5$), 9.5 (s, 1H, -NH), 12.6 (s, 1H, -NH); m/z (EI), 267 (M^+ , 93%), 222 ($[M-CONH_3]^+$).

138 2-(3'-hydroxyphenyl)-1H-benzimidazole-4-carboxylate³⁰³

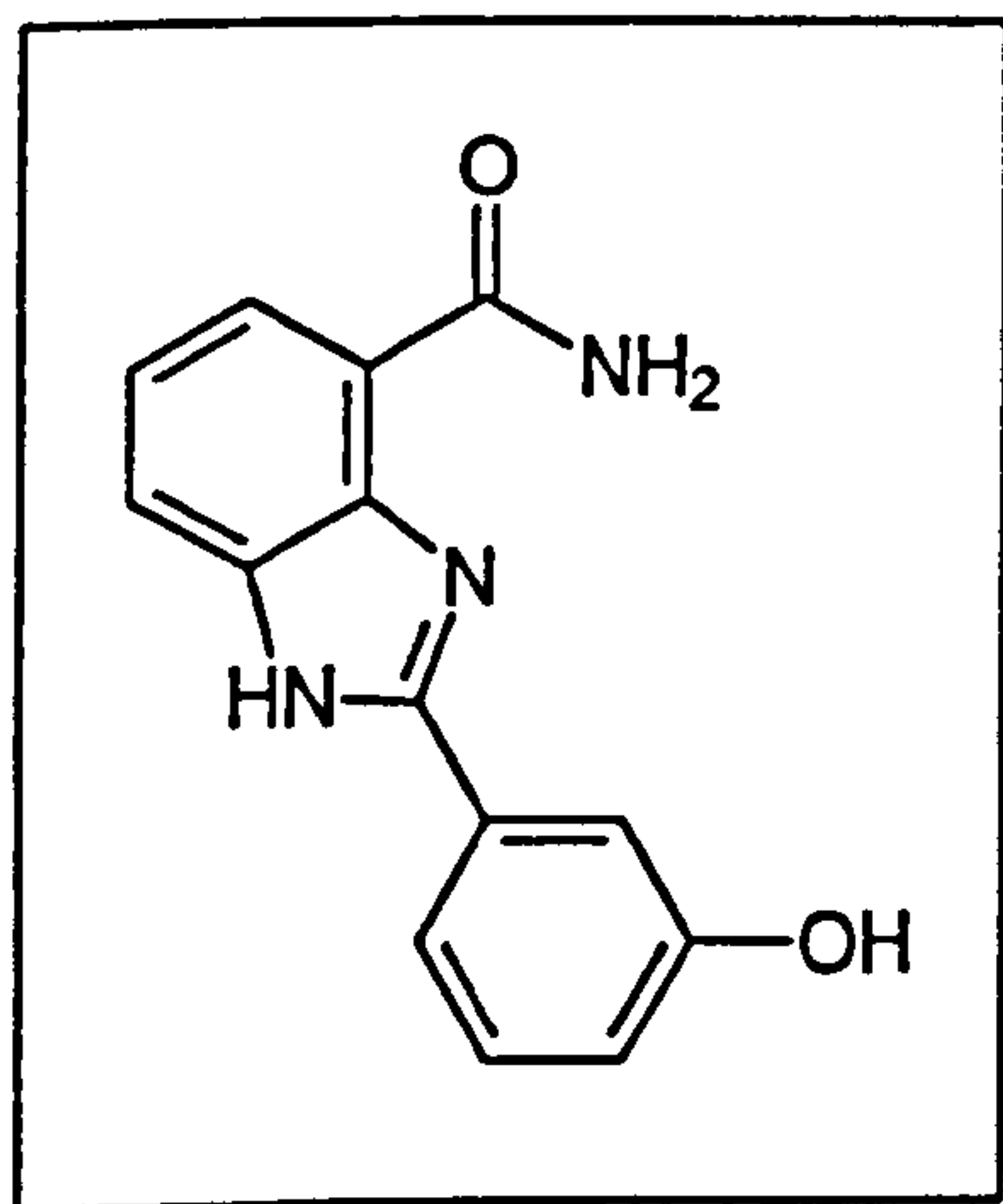


Prepared according to Standard Procedure E from 3-nitroanthranilic acid 103 (1.0 g, 5.5 mmol) and 3-hydroxybenzaldehyde (0.9 g, 7.7 mmol) to give the product as a brown solid.

Yield : 0.93 g, 3.7 mmol, 67%, m.p. 329-330 °C.

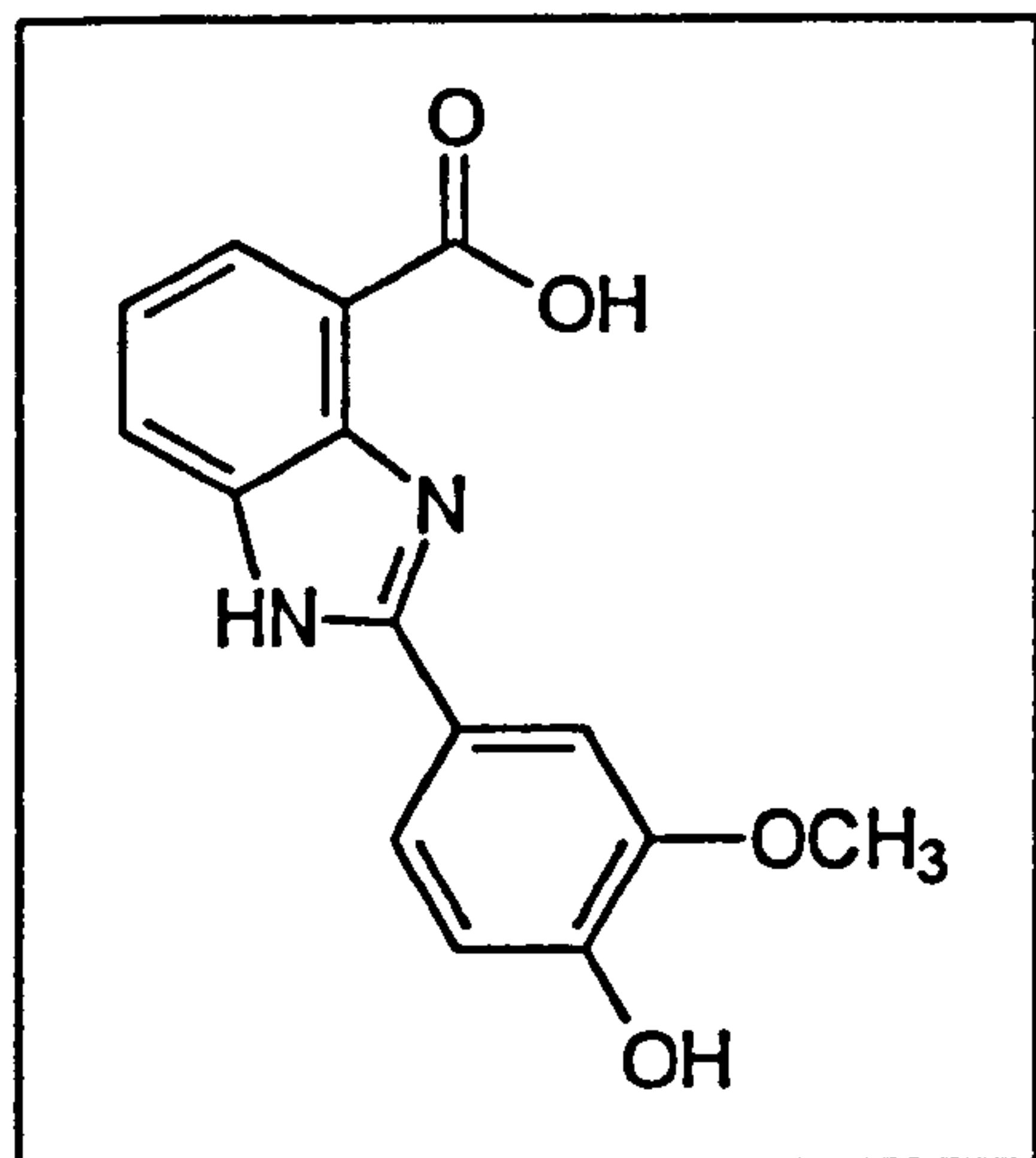
$\nu_{\max}/\text{cm}^{-1}$ 3369 (br, OH), 1632 and 1596 (CO_2H), 1491 ($\text{C}=\text{O}$); δ_{H} (200 MHz, d_6 -DMSO) 7.01-7.04 (d, 1H, Ar-4H), 7.42-7.44 (m, 2H, Ar-5H, Ph-4'H), 7.81-8.01 (m, 4H, Ar-6H, Ph-3'/5'/6'H); m/z (EI) 254 (M^+ , 55 %) 236 ($[M-\text{H}_2\text{O}]^+$), 208 ($[M-\text{CH}_2\text{O}_2]^+$).

139 2-(3'-hydroxyphenyl)-1H-benzimidazole-4-carboxamide



Prepared according to Standard Procedure F from 2-(3'-hydroxyphenyl)-1H-benzimidazole-4-carboxylate 138 (0.5 g, 2.0 mmol). The product was purified by column chromatography using DCM:MeOH (9:1) as eluent, followed by recrystallisation from methanol to give a white crystalline solid (0.46 g, 1.8 mmol, 92%), m.p. 294-296 °C.

Anal. found C 66.64, H 4.02, N 16.41, $\text{C}_{14}\text{H}_{11}\text{N}_3\text{O}_2$ requires C 66.40, H 4.38, N 16.59%; $\nu_{\max}/\text{cm}^{-1}$ 3411 and 3332 (1° amide NH), 3190 (br, OH), 1661 and 1600 ($\text{C}=\text{O}$); δ_{H} (200 MHz, d_6 -DMSO) 7.02-7.07 (dd, 1H, Ar-4H), 7.40-7.53 (m, 2H, Ar-5H, Ph-6'H), 7.73-7.84 (m, 3H, -NH, Ph-4'/5'H), 7.95-7.99 (d, 2H, Ar-6H, Ph-2'H), 9.50 (s, 1H, OH), 9.95 (br, s 1H, -NH), 13.45 (br s, 1H, -NH); m/z (EI) 253 (M^+ , 100%), 236 ($[M-\text{NH}_3]^+$), 208 ($[M-\text{CONH}_3]^+$).

140 2-(3'-Methoxy-4'-hydroxyphenyl)-1H-benzimidazole-4-carboxylate³⁰³

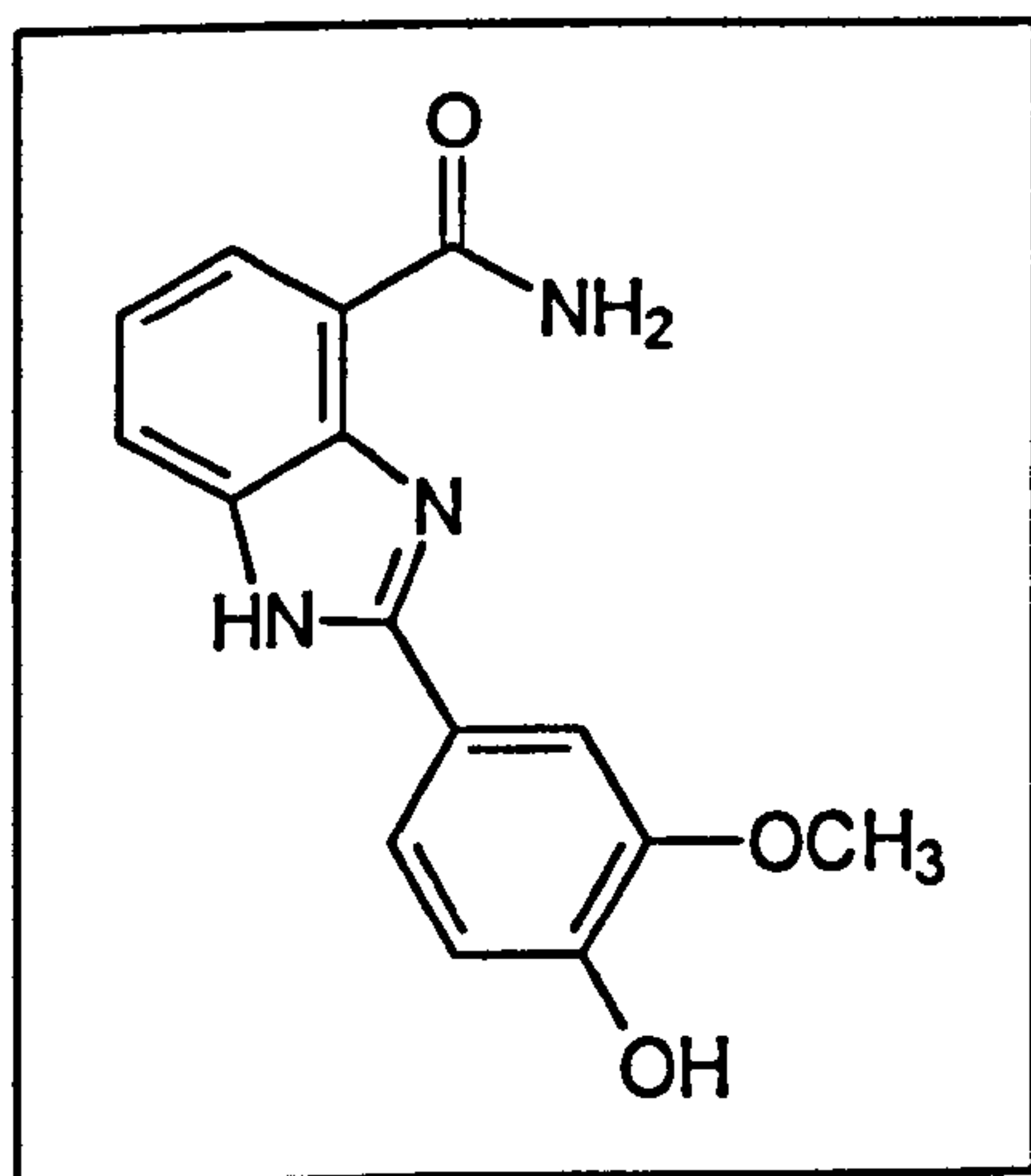
Prepared according to Standard Procedure E, from 3-nitroanthranilic acid 103 (1.0 g, 5.5 mmol) and 3-methoxy-4-hydroxybenzaldehyde (1.2 g, 7.7 mmol) to give a pale orange solid.

Yield : 0.69 g, 2.4 mmol, 44%, m.p. 299-302 °C.

Anal. found C 63.48, H 4.07, N 9.57, $C_{15}H_{12}N_2O_4$ requires C 63.37, H 4.26, N 9.86%; $\nu_{\max}/\text{cm}^{-1}$ 3519 (br, OH), 3075 (br, NH), 2847 (OCH_3), 1600 (CO_2H); δ_{H} (200 MHz, d_6 -DMSO)

4.00 (s, 3H, 3'- OCH_3), 6.99-7.03 (d, 1H, Ar-4H, $J = 8.2$), 7.34-7.42 (t, 1H, Ar-5H, $J = 7.8$), 7.85-7.97 (m, 5H, Ar-6H, -NH, Ph-2'/5'/6'H), 9.8 (br s, 1H, -OH); m/z (EI) 284 (M^+ , 99%), 266 ($[M-\text{H}_2\text{O}]^+$), 238 ($[M-\text{CH}_2\text{O}_2]^+$).

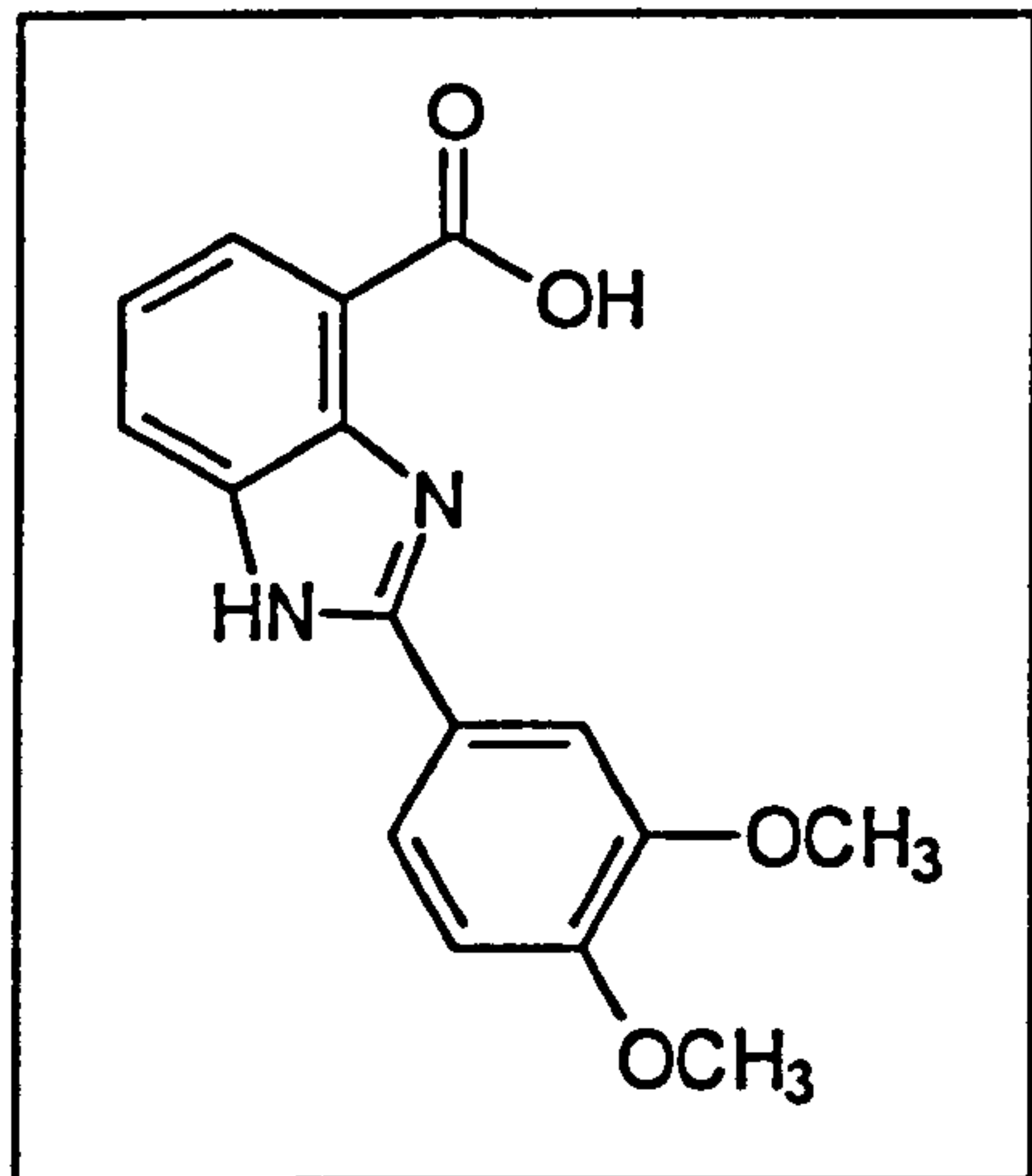
141 2-(3'-Methoxy-4'-hydroxyphenyl)-1H-benzimidazole-4-carboxylamide



Prepared according to Standard Procedure F, from 2-(3'-methoxy-4'-hydroxyphenyl)-1H-benzimidazole-4-carboxylate 141 (.15 g, 0.5 mmol). The product was recrystallised from methanol to give orange crystals (0.07 g, 0.2 mmol, 46%), m.p. 244-245 °C.

Anal. found C 61.13, H 5.38, N 13.37, $C_{15}H_{13}N_3O_3 \cdot 1 \text{ mol. CH}_3\text{OH}$ requires C 60.94, H 5.43, N 13.33%; $\nu_{\max}/\text{cm}^{-1}$ 3379 (br, OH), 3195 and 3001 (br, 1 ° amide NH), 2838 (OCH_3),

1659 ($\text{C}=\text{O}$), 1598 ($\text{C}=\text{O}$); δ_{H} (200 MHz, d_6 -DMSO) 4.01 (s, 3H, - OCH_3), 7.04-7.08 (d, 1H, Ar-4H, $J = 8.2$), 7.35-7.39 (t, 1H, Ar-5H, $J = 7.8$), 7.76-7.81 (m, 4H, Ph-2'/5'/6'H, -NH), 7.91-7.95 (d, 1H, Ar-6H, $J = 7.5$), 9.5 (s, 1H, -NH), 9.8 (s, 1H, -OH), 13.2 (s, 1H, -NH); m/z (EI) 283 (M^+ , 100%), 266 ($[M-\text{NH}_3]^+$), 238 ($[M-\text{CONH}_2]^+$).

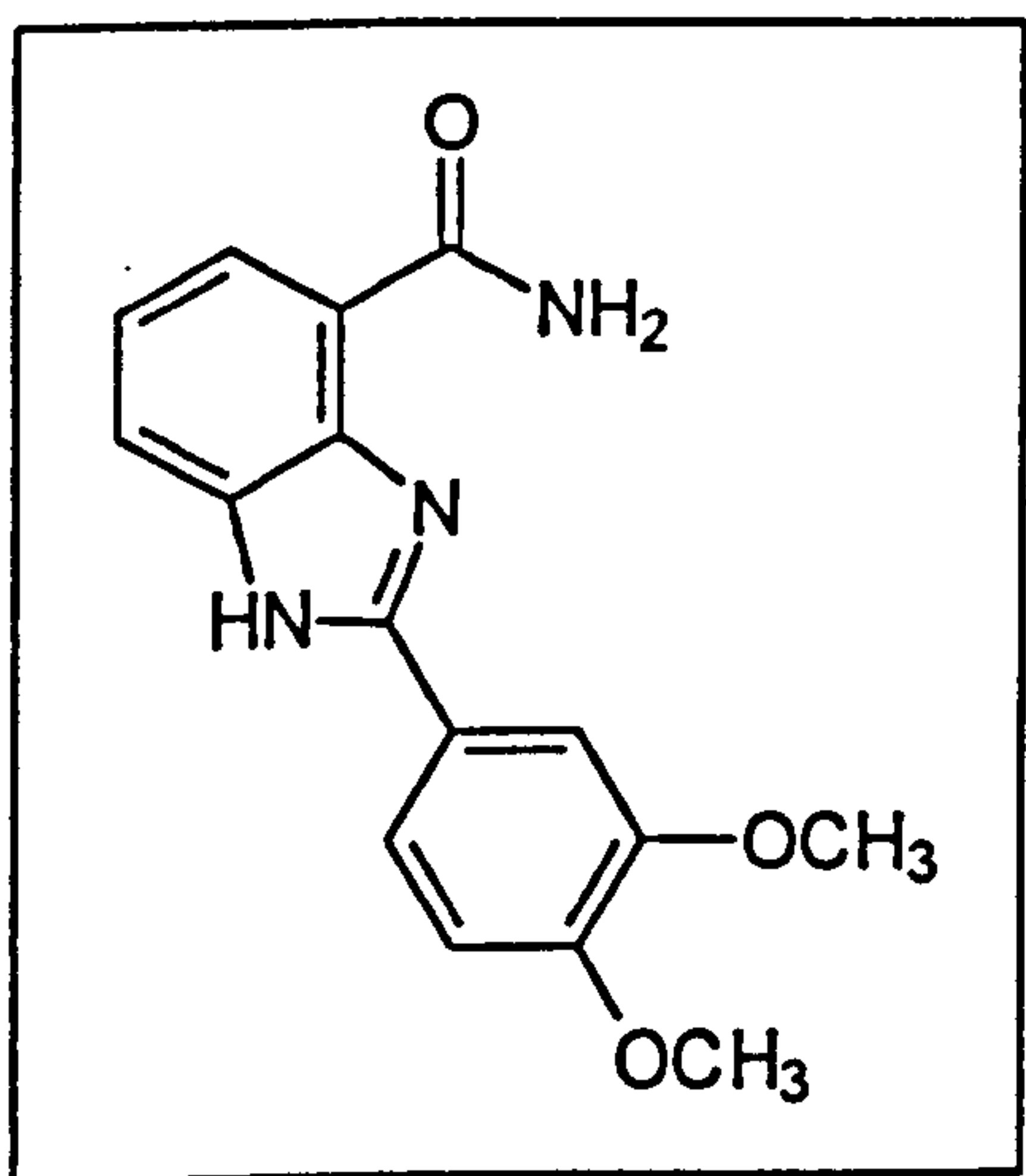
142 2-(3',4'-Dimethoxyphenyl)-1*H*-benzimidazole-4-carboxylate³⁰³

Prepared according to Standard Procedure E, from 3-nitroanthranilic acid 103 (0.75 g, 4.1 mmol) and 3,4-dimethoxybenzaldehyde (0.96 g, 5.7 mmol) to give a pale brown solid.

Yield : 0.57 g, 1.9 mmol, 46%.

Anal. found C 57.45, H 4.16, N 8.18, $C_{16}H_{14}N_2O_4 \cdot 1$ mol. HCl requires C 57.40, H 4.52, N 8.37%; $\nu_{\max}/\text{cm}^{-1}$ 3079 (NH), 2839 (OCH₃), 1604 (CO₂H); δ_H (200 MHz, d_6 -DMSO) 3.95-

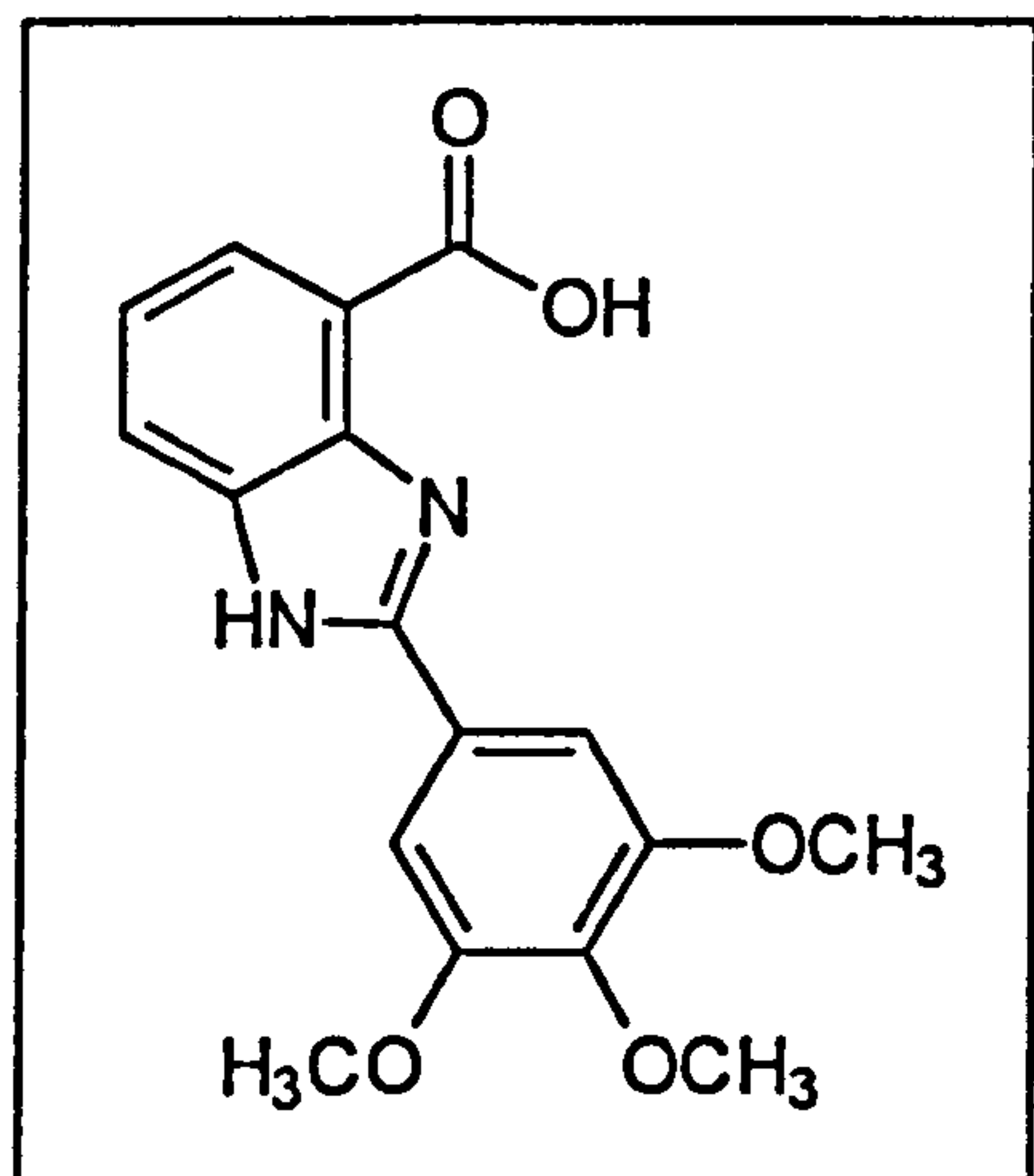
4.01 (d, 6H, 3'/4'-OCH₃), 7.21-7.25 (d, 1H, Ar-4H), 7.41 (t, 1H, Ar-5H), 7.88-8.02 (m, 4H, Ar-6H, Ph-2'/5'/6'H); m/z (EI) 298 (M^+ , 77%), 280 ($[M-H_2O]^+$), 236 ($[M-(CH_3)_2]^+$).

143 2-(3',4'-Dimethoxyphenyl)-1*H*-benzimidazole-4-carboxamide

Prepared according to Standard Procedure F, from 2-(3',4'-dimethoxyphenyl)-1*H*-benzimidazole-4-carboxylate 142 (0.2 g, 0.7 mmol). The product was recrystallised from methanol to give cream crystals (0.06 g, 0.2 mmol, 31%), m.p. 267-269 °C.

Anal. found C 60.93, H 4.58, N 13.37, $C_{16}H_{15}N_3O_3 \cdot 0.5$ mol. HCl requires C 60.90, H 4.95, N 13.32%; $\nu_{\max}/\text{cm}^{-1}$ 3422 (NH), 2834 and 2777 (OCH₃), 1648 and 1603 (C=O); δ_H

(200 MHz, d_6 -DMSO) 3.96-4.03 (d, 6H, 3'/4'-OCH₃), 7.26-7.30 (d, 1H, Ar-4H, $J = 8.2$), 7.39-7.46 (t, 1H, Ar-5H, $J = 7.8$), 7.80-7.98 (m, 5H, Ar-6H, Ph-2'/5'/6'H, -NH), 9.5 (s, 1H, -NH), 13.3 (s, 1H, -NH); m/z (EI) 297 (M^+ , 100%), 280 ($[M-NH_3]^+$), 254 ($[M-CONH_3]^+$).

144 2-(3',4',5'-Trimethoxyphenyl)-1H-benzimidazole-4-carboxylate³⁰³

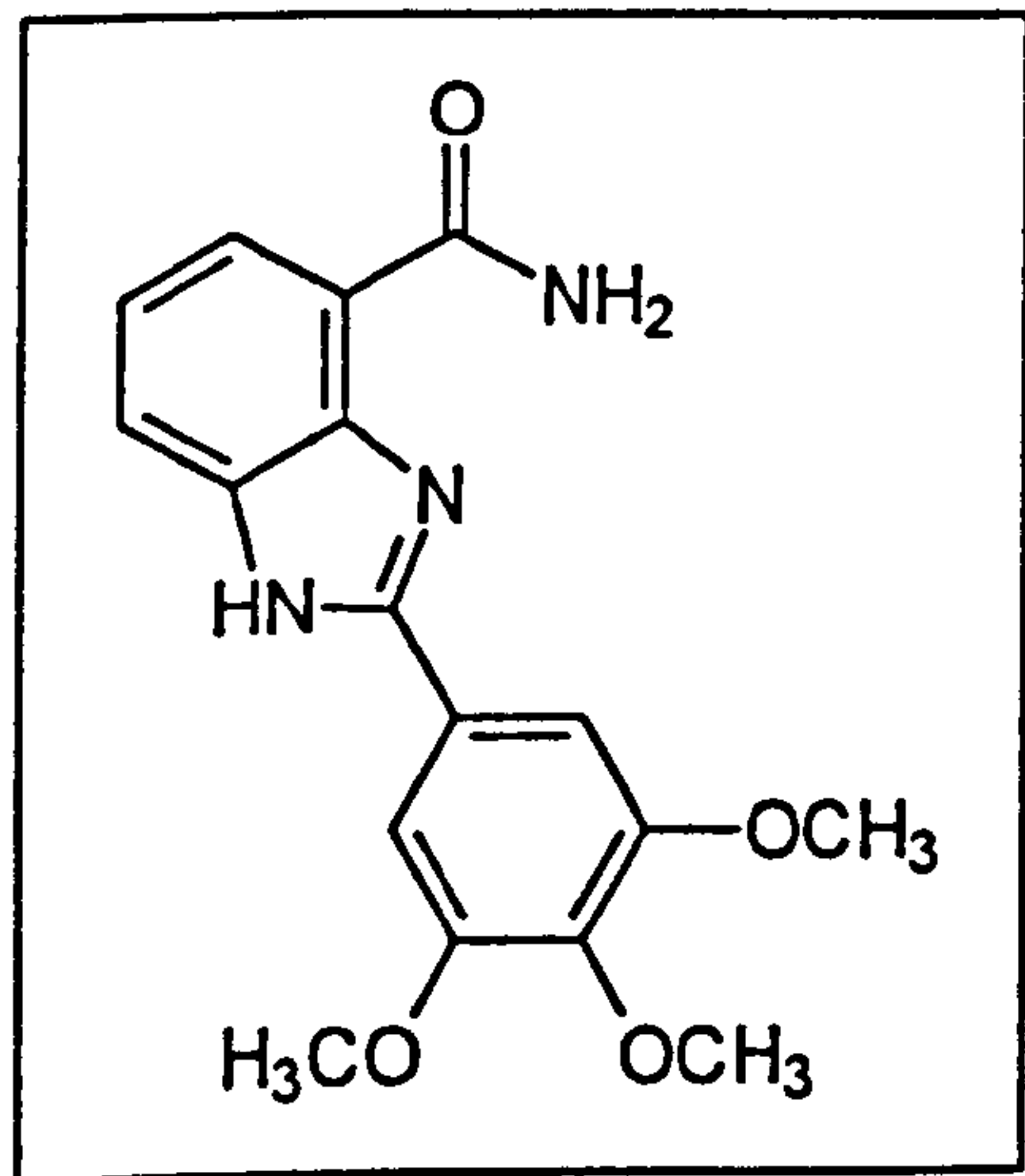
Prepared according to Standard Procedure E, from 3-nitroanthranilic acid 103 (0.75 g, 4.1 mmol) and 3,4,5-trimethoxybenzaldehyde (1.13 g, 5.7 mmol) to give a pale brown solid.

Yield : 0.54 g, 1.7 mmol, 40%, m.p. 293-296 °C.

Anal. found C 61.94, H 4.86, N 8.08, C₁₇H₁₆N₂O₅·0.1 mol. CH₃OH requires C 61.95, H 4.99, N 8.45%; $\nu_{\max}/\text{cm}^{-1}$ 3260 (br, NH), 2937 and 2833 (OCH₃), 1593 (CO₂H); δ_{H} (200

MHz, *d*₆-DMSO) 3.84 (s, 3H, 4'-OCH₃), 4.02 (s, 6H, 3'/5'-OCH₃), 7.50 (t, 1H, Ar-5H), 7.84 (s, 2H, Ph-2'/6'H), 7.95-7.99 (d, 1H, Ar-4H), 8.04-8.08 (d, 1H, Ar-6H); *m/z* (EI) 328 (M⁺, 27%), 282 ([M-CH₂O₂]⁺).

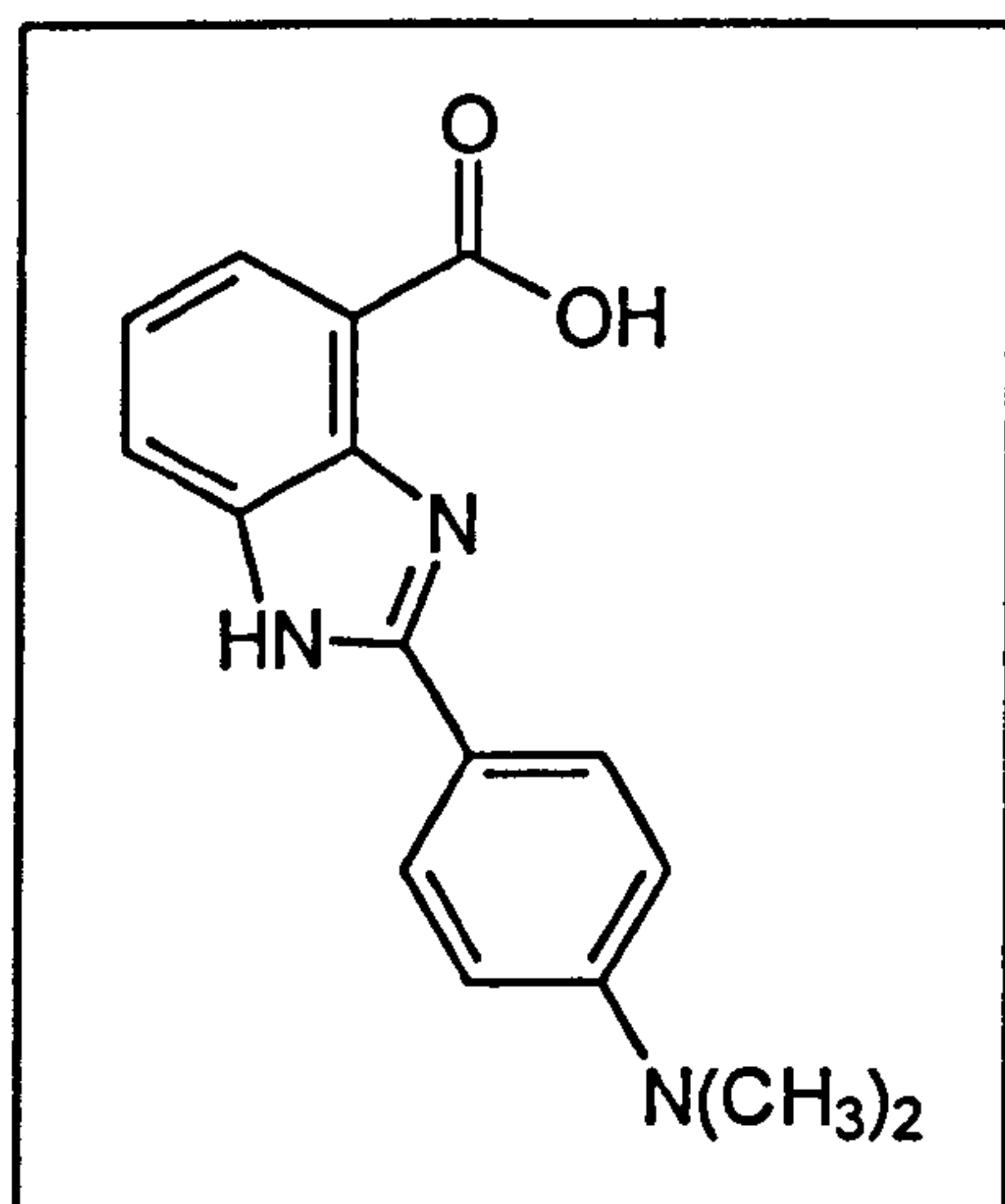
145 2-(3',4',5'-Trimethoxyphenyl)-1H-benzimidazole-4-carboxamide



Prepared according to Standard Procedure F, from 2-(3',4',5'-trimethoxyphenyl)-1H-benzimidazole-4-carboxylate 144 (0.2 g, 0.6 mmol). The product was obtained as a pale brown crystalline solid after recrystallisation from methanol/water (0.06 g, 0.18 mmol, 30%), m.p. 275-276 °C.

Anal. found C 62.20, H 5.07, N 12.54, C₁₇H₁₇N₃O₄ requires C 62.38, H 5.23, N 12.84%; $\nu_{\max}/\text{cm}^{-1}$ 3436 (NH), 3258 and 3179 (br, 1° amide NH), 2927 and 2843 (OCH₃), 1646 and

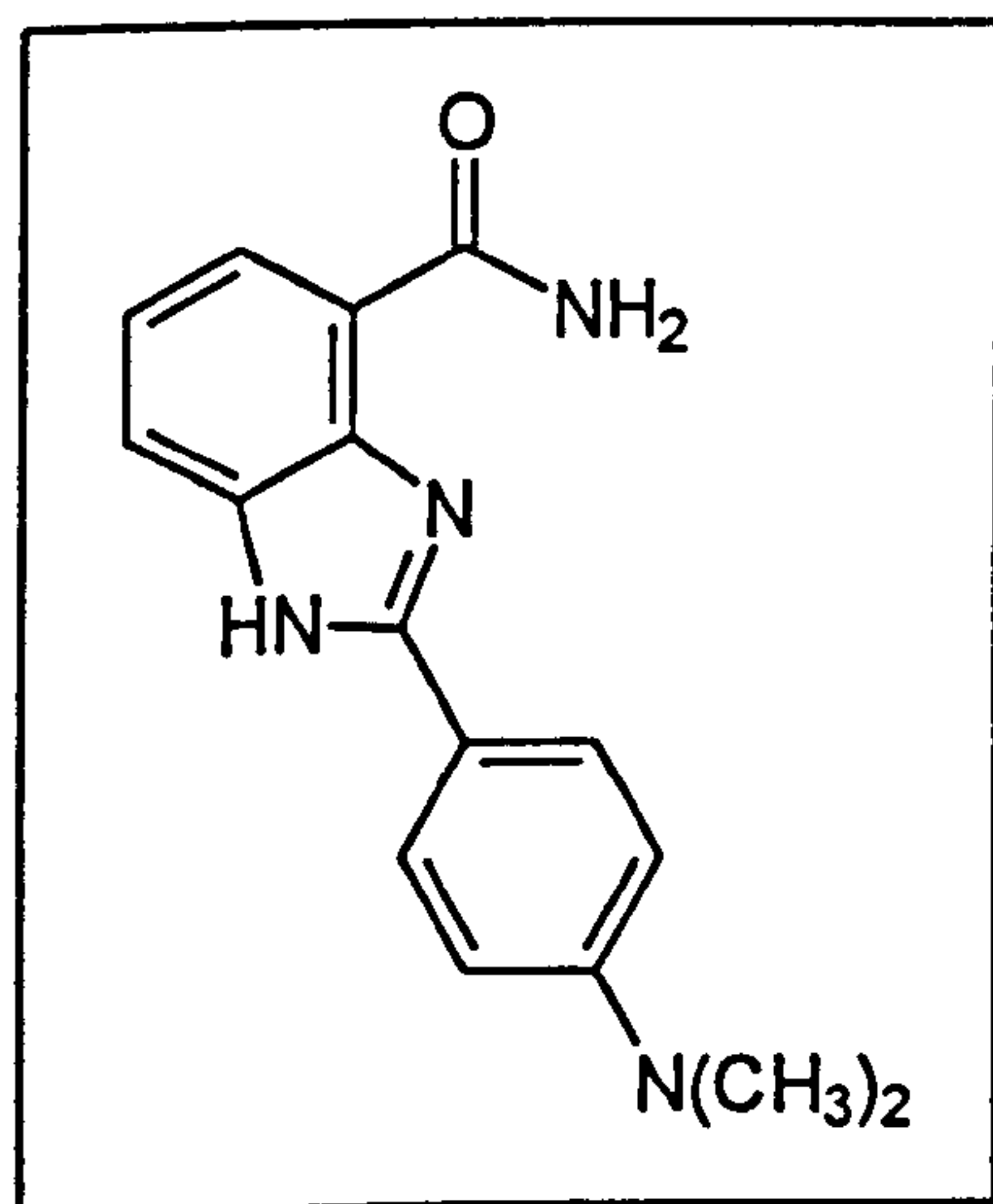
(C=O); δ_{H} (200 MHz, *d*₆-DMSO) 3.84 (s, 3H, 4'-OCH₃), 4.02 (s, 6H, 3'/5'-OCH₃), 7.40-7.86 (t, 1H, Ar-5H, *J* = 7.8), 7.82-7.86 (s, 2H, Ph-2'/6'H), 7.82-7.86 (d, 2H, -NH, Ar-4H, *J* = 7.7), 7.95-7.91 (d, 1H, Ar-6H, *J* = 7.5), 9.6 (br s, 1H, -NH), 13.4 (br s, 1H, -NH); *m/z* (EI) 327 (M⁺, 98%), 310 ([M-NH₃]⁺), 295 ([M-CH₃OH]⁺), 284 ([M-CONH]⁺).

146 2-(4'-*N,N*-dimethylaminophenyl)-1*H*-benzimidazole-4-carboxylate³⁰³

Prepared according to Standard Procedure E, from 3-nitroanthranilic acid 103 (0.75 g, 4.1 mmol) and 4-*N,N*-dimethylaminobenzaldehyde (0.86 g, 5.7 mmol). The crude green product was used without further purification (0.56 g, 2.0 mmol, 48%), m.p. >360 °C (lit. m.p.³⁰³ >360 °C).

Anal. found C 66.37, H 5.39, N 13.23, $C_{16}H_{13}N_3O_2 \cdot 0.65$ mol. CH_3OH requires C 66.18, H 5.87, N 13.76%; ν_{max}/cm^{-1} 3436 (NH), 3258 and 3179 (1° amide NH), 2927 and 2843 (CH_3),

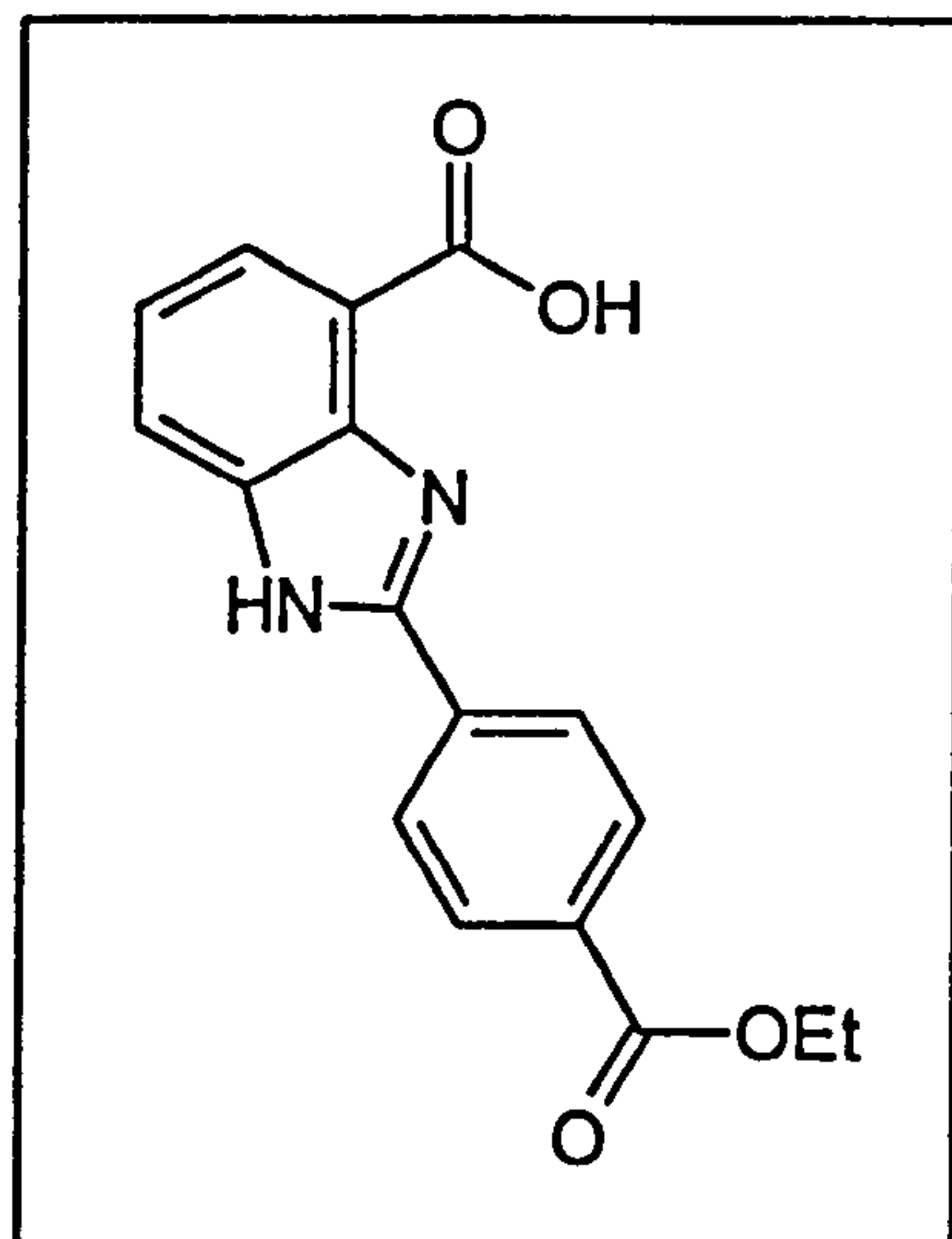
1646 and 1603 (C=O); δ_H (200 MHz, d_6 -DMSO) 3.12 (s, 6H, $2 \times N-CH_3$), 6.91-6.96 (d, 2H, Ph-2'/6'H, $J = 8.9$), 7.37-7.41 (t, 1H, Ar-5H, $J = 7.6$), 7.82-7.86 (d, 1H, Ar-4H, $J = 7.7$), 7.88-7.92 (d, 1H, Ar-6H, $J = 7.5$), 8.21-8.25 (d, 2H, Ph-3'/5'H, $J = 8.8$); m/z (EI) 327 (M^+ , 100%) 310 ($[M-NH_3]^+$).

147 2-(4'-*N,N*-dimethylaminophenyl)-1*H*-benzimidazole-4-carboxylamide

Prepared according to Standard Procedure F, from 2-(4'-*N,N*-dimethylaminophenyl)-1*H*-benzimidazole-4-carboxylate 146 (0.2 g, 0.7 mmol). The product was purified by column chromatography, using DCM:MeOH (9:1) as eluent, followed by recrystallisation from methanol to give a brown crystalline solid (0.07 g, 0.3 mmol, 36%), m.p. 281-283 °C.

Anal. found C 68.32, H 5.54, N 19.81, $C_{16}H_{16}N_4O$ requires C 68.55, H 5.75, N 19.99%; ν_{max}/cm^{-1} 3302 and 3167 (1° amide

NH), 2817 (CH_3), 1660 and 1610 (C=O); δ_H (200 MHz, d_6 -DMSO) 3.12 (s, 6H, $2 \times N-CH_3$), 6.94-6.98 (d, 2H, Ph-2'/6'H, $J = 9.0$), 7.31-7.39 (t, 1H, Ar-5H, $J = 7.8$), 7.72-7.76 (d, 1H, Ar-4H, $J = 7.9$), 7.82-7.83 (br s, 1H, -NH), 7.88-7.92 (d, 1H, Ar-6H, $J = 7.6$), 8.13-8.17 (d, 2H, 3'/5'H, $J = 8.9$); m/z (EI) 280 (M^+ , 100%), 263 ($[M-NH_3]^+$), 235 ($[M-CONH_2]^+$).

148 2-(4'-Ethoxycarbonylphenyl)-1H-benzimidazole-4-carboxylate³⁰³

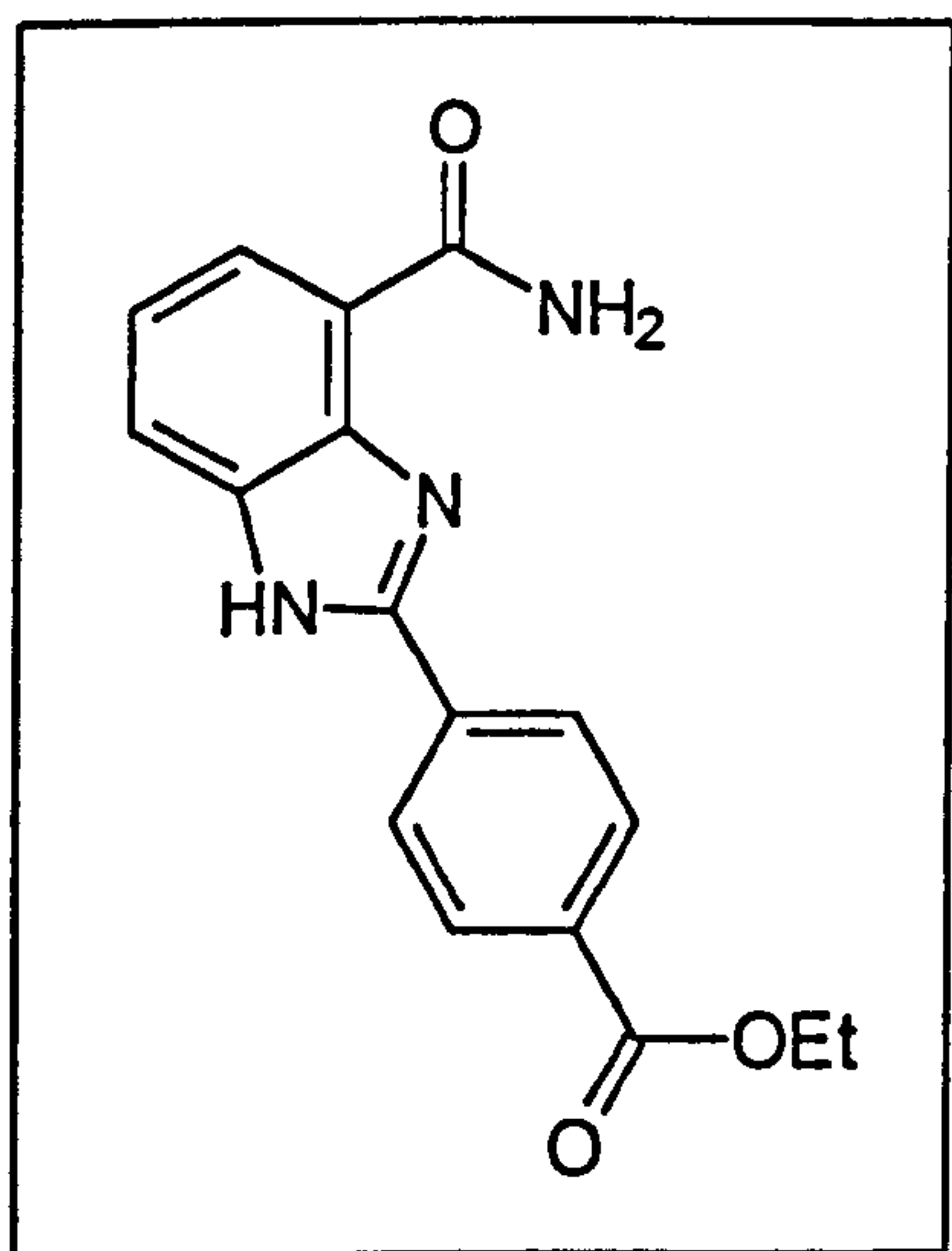
Prepared according to Standard Procedure E, from 3-nitroanthranilic acid 103 (1 g, 5.5 mmol) and 4-benzyloxycarbonylbenzaldehyde 156 (1.8 g, 7.7 mmol) to give a pale orange solid.

Yield : 0.52 g, 1.7 mmol, 31%, m.p. 278 °C.

$\nu_{\max}/\text{cm}^{-1}$ 3297 (br, OH), 1721 (C=O); δ_{H} (200 MHz, d_6 -DMSO) 1.41-1.48 (t, 3H, CH_2CH_3 , $J = 7.1$), 4.40-4.50 (q, 2H, CH_2CH_3 , $J = 7.1$), 5.49-7.58 (m, 2H, Ar-5H, -NH), 7.93-7.96 (d, 1H, Ar-4H, $J = 7.4$), 8.02-8.06 (d, 1H, Ar-6H, $J = 8.2$),

8.17-8.24 (m, 2H, Ph-2'/6'H, $J = 8.2$), 8.55-8.59 (d, 2H, Ph-3'/5'H, $J = 8.2$); m/z (EI) 310 (M^+ , 100%), 292 ($[\text{M}-\text{H}_2\text{O}]^+$), 265 ($[\text{M}-\text{CH}_2\text{O}_2]^+$).

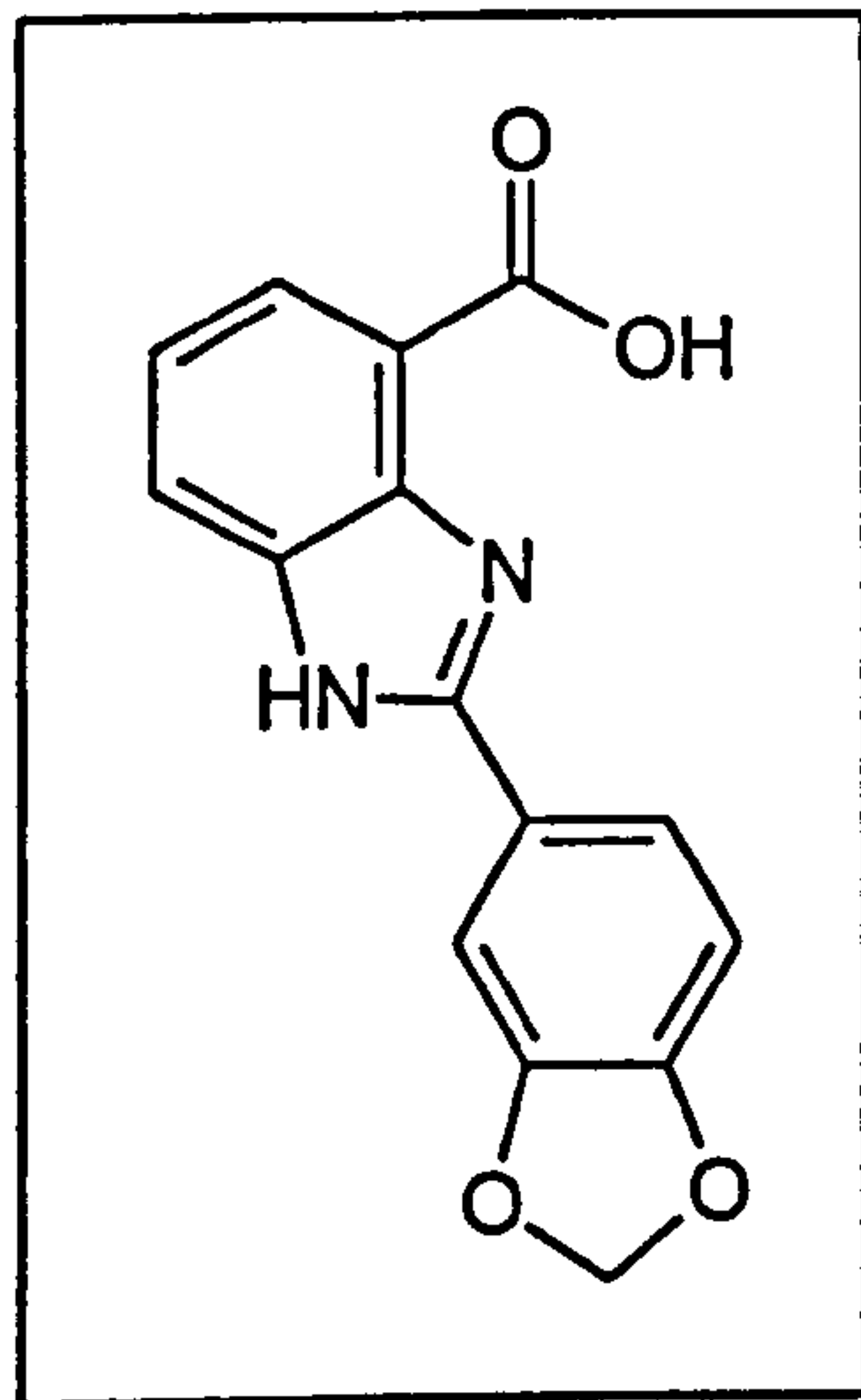
149 2-(4'-Ethoxycarbonylphenyl)-1H-benzimidazole-4-carboxamide



Prepared according to Standard Procedure F from 2-(4'-ethoxycarbonylphenyl)-1H-benzimidazole-4-carboxylate 148 (0.2 g, 0.6 mmol). The product was purified by column chromatography using DCM:MeOH (9:1) as eluent followed by recrystallisation from EtOH to give a peach coloured solid (0.13 g, 0.4 mmol, 66%), m.p. 110-112 °C.

Anal. found C 66.13, H 4.63, N 12.99, $\text{C}_{17}\text{H}_{13}\text{N}_3\text{O}_3 \cdot 0.1 \text{ mol. CH}_3\text{CH}_2\text{OH}$ requires C 65.80, H 5.01, N 13.39%; $\nu_{\max}/\text{cm}^{-1}$ 3380 (1° amide NH), 1691 and 1654 (C=O); δ_{H} (200 MHz,

d_6 -DMSO) 1.42-1.49 (t, 3H, OCH_2CH_3 , $J = 7.1$), 4.41-4.51 (q, 2H, OCH_2CH_3 , $J = 7.1$), 7.45-7.53 (t, 1H, Ar-5H), 7.86-8.02 (m, 3H, Ar-4/6H, -NH), 8.23-8.27 (d, 2H, Ph-2'/6'H, $J = 8.4$), 8.47-8.51 (d, 2H, Ph-3'/5'H, $J = 8.3$), 9.40 (s, 1H, -NH), 13.74 (s, 1H, -NH); m/z (EI) 309 (M^+ , 100%), 292 ($[\text{M}-\text{NH}_3]^+$), 264 ($[\text{M}-\text{CONH}_3]^+$), 236 ($[\text{M}-\text{CO}_2\text{CH}_2\text{CH}_3]^+$).

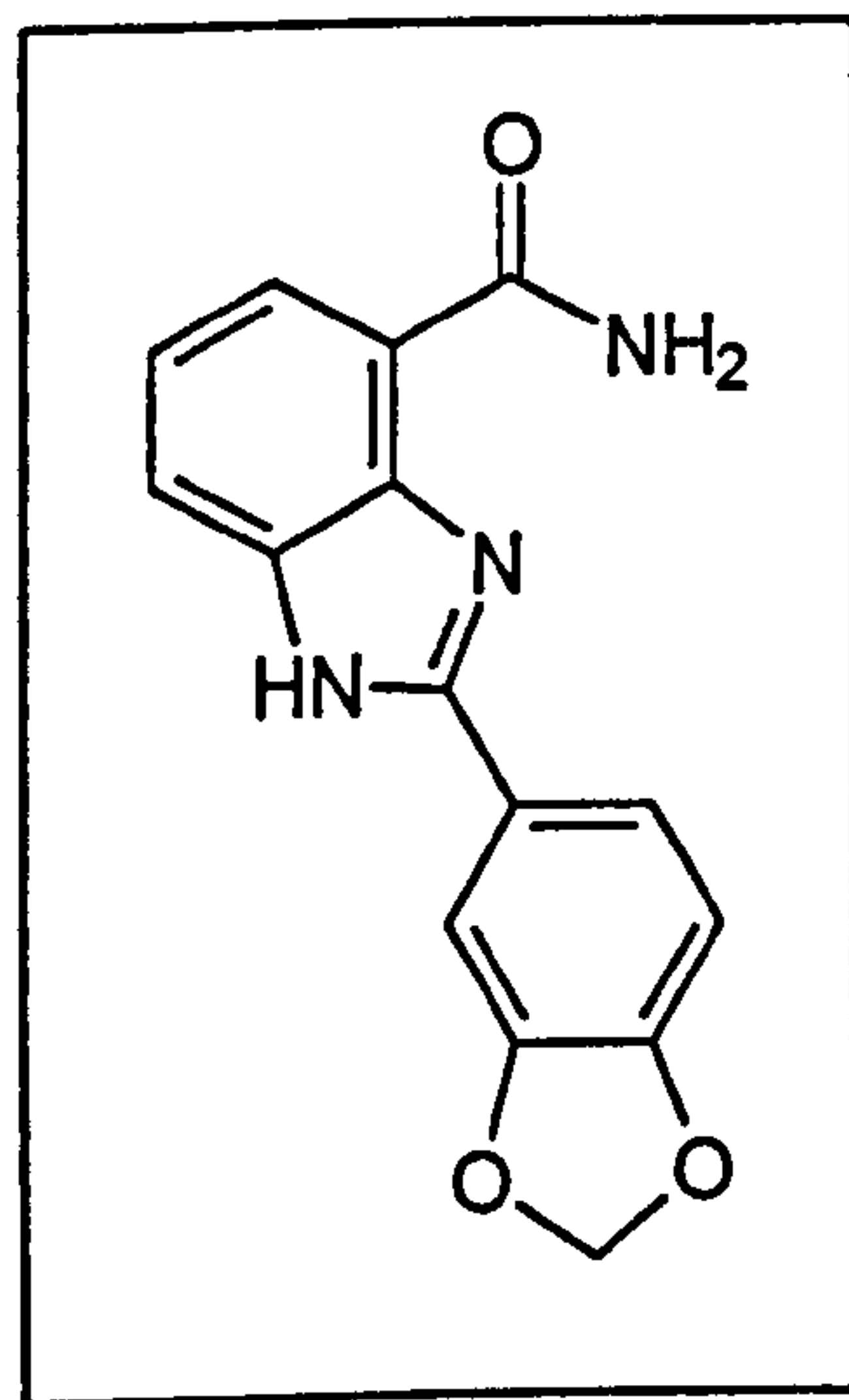
150 2-(3',4'-Methylenedioxyphenyl)-1H-benzimidazole-4-carboxylate³⁰³

Prepared according to Standard Procedure E from 3-nitroanthranilic acid 103 (1 g, 5.5 mmol) and piperonaldehyde (1.15 g, 7.7 mmol). The product was purified by column chromatography using DCM:MeOH (9:1) as eluent to give a brown solid (0.51 g, 1.8 mmol, 33%), m.p. 304-306 °C.

Anal. found C 61.76, H 3.23, N 9.31 $C_{15}H_{10}N_2O_4 \cdot 0.5 \text{ mol. } H_2O$ requires C 61.85, H 3.81, N 9.62%; $\nu_{\max}/\text{cm}^{-1}$ 3270 (br, OH), 2786 (O-CH₂-O), 1503 (C=O), 1480 (CO₂H), 1249 (C-O); δ_{H} (200 MHz, *d*₆-DMSO) 6.22 (s, 2h, O-CH₂-O), 7.15-7.19 (d, 1h, Ar-4H, *J* = 7.9),

7.34-7.41 (t, 1H, Ar-5H, *J* = 7.8), 7.86-7.99 (m, 4H, Ar-6H, Ph-2'/3'/6'H).

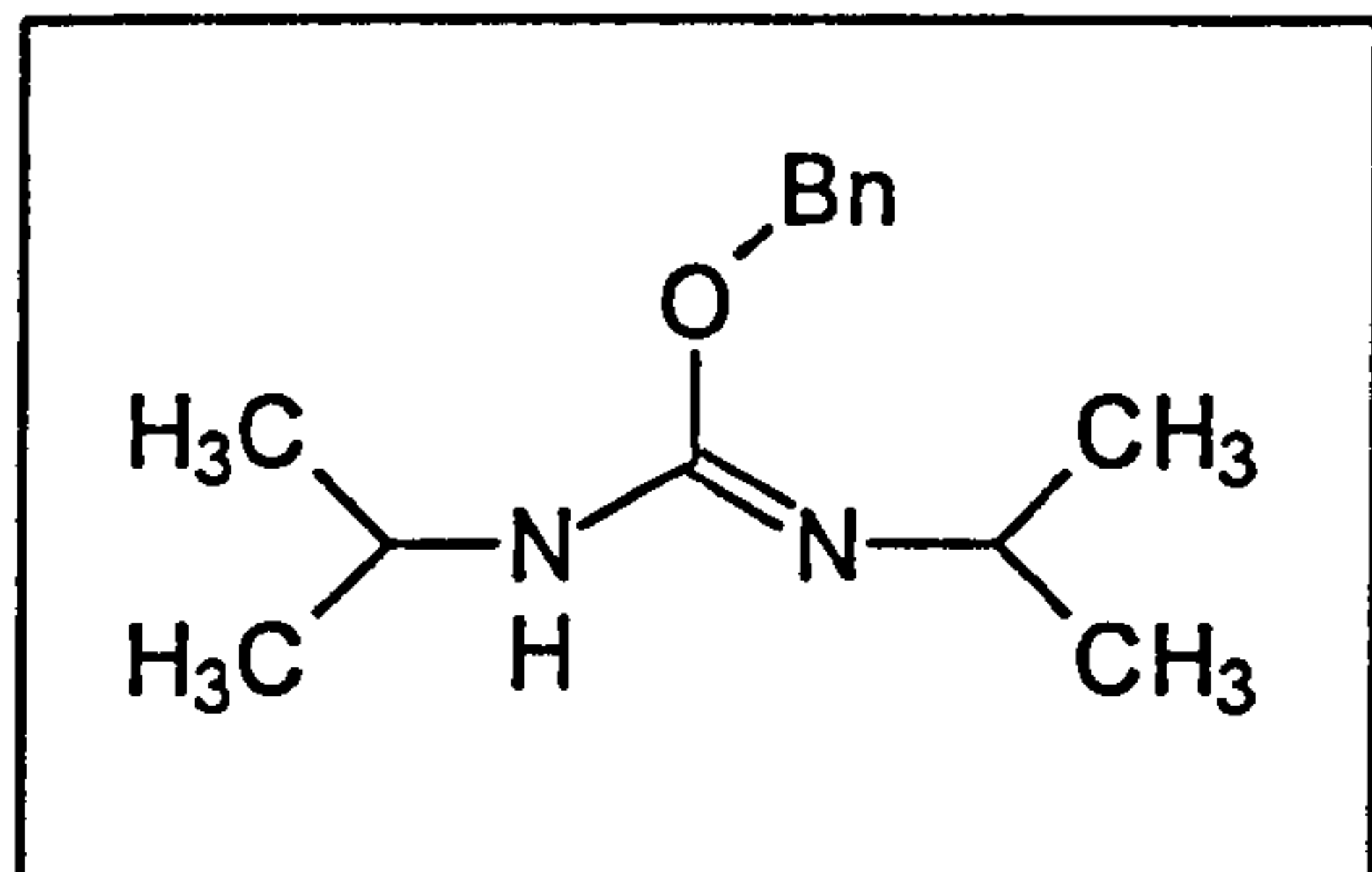
151 2-(3',4'-Methylenedioxyphenyl)-1H-benzimidazole-4-carboxylamide



Prepared according to Standard Procedure F from 2-(3',4'-methylenedioxyphenyl)-1H-benzimidazole-4-carboxylate 150 (0.2 g, 0.7 mmol). The product was purified by column chromatography using DCM:MeCN (8:2) as eluent to give a cream crystalline solid (0.09 g, 0.3 mmol, 46%), m.p. 286-288 °C.

Anal. found C 64.58, H 3.75, N 14.83, $C_{15}H_{11}N_3O_3$ requires C 64.05, H 3.94, N 14.94%; $\nu_{\max}/\text{cm}^{-1}$ 3345 and 3144 (1° amide NH), 2788 (O-CH₂-O), 1650 (C=O), 1602 (CO₂H), 1245 (C-O); δ_{H} (200 MHz, *d*₆-DMSO) 6.25 (s, 2H, O-CH₂-O), 7.21-7.26 (d, 1H, Ar-6H, *J* =

8.4), 7.37-7.45 (t, 1H, Ar-5H, *J* = 7.8), 7.78-7.97 (m, 5H, Ar-4H, Ph-2'/5'/6'H, -NH); *m/z* (EI) 282 (*M*⁺, 25%), 264 ([*M*-H₂O]⁺), 236 ([*M*-CH₂O₂]⁺).

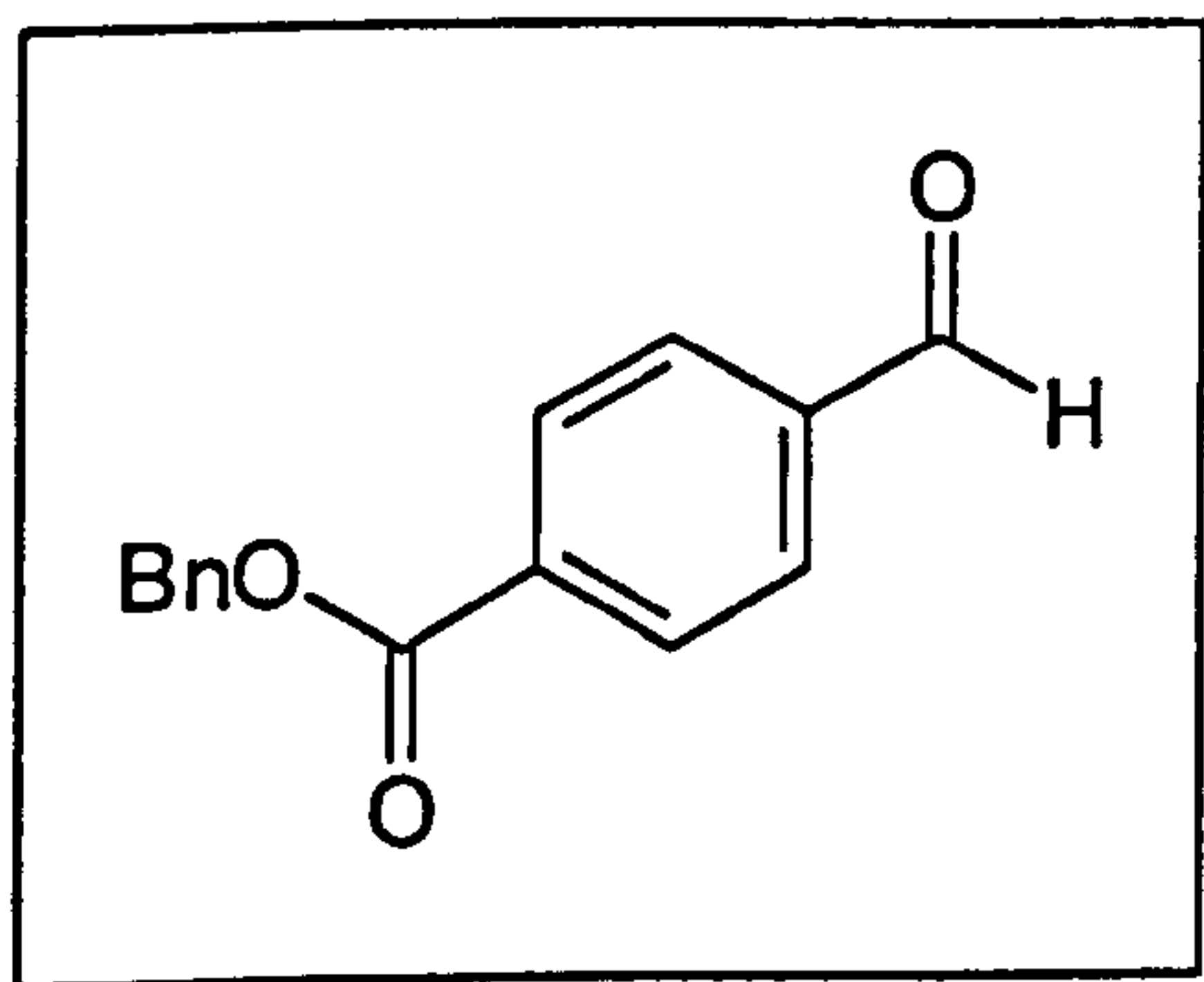
155 *N,N*-Diisopropyl-*O*-benzyl isourea³⁰⁵

Benzyl alcohol (5.0 g, 0.05 M) was added dropwise, over a period of 10 minutes, to a stirred solution of *N,N*-diisopropylcarbodiimide (5.9 g, 0.05 M) containing copper (I) chloride (17 mg), at 0 °C. The reaction mixture was stirred for a further 1 hour at 0 °C and then

allowed to warm up to room temperature, and stirred overnight. The volume of the mixture was doubled with petrol (40/60) and the whole was applied to a filter pad of neutral alumina, to remove the copper salts. The pad was washed with an excess of petrol (40/60). The solvent was removed from the filtrate under reduced pressure, and the remaining colourless oil was dried under high vacuum (7.7 g, 0.03 M, 71%).

$\nu_{\max}/\text{cm}^{-1}$ 2965 (NH), 1666 (C=N); δ_{H} (200 MHz, d_6 -DMSO) 1.10-1.13 (d, 12H, $2 \times \text{CH}(\text{CH}_3)_2$), 3.19 (m, 3H, $2 \times \text{CH}_2\text{-NH}$), 5.09 (s, 2H, $\text{CH}_2\text{-Ph}$), 7.24-7.39 (m, 5H, $\text{Ph-2'/3'/4'/5'/6'H}$); m/z (EI) 234 (M^+ , 9%), 219 ($[\text{M}-\text{CH}_3]^+$), 191 ($[\text{M}-\text{CH}(\text{CH}_3)_2]^+$), 91 (Bn^+).

156 4-Benzoyloxycarbonylbenzaldehyde

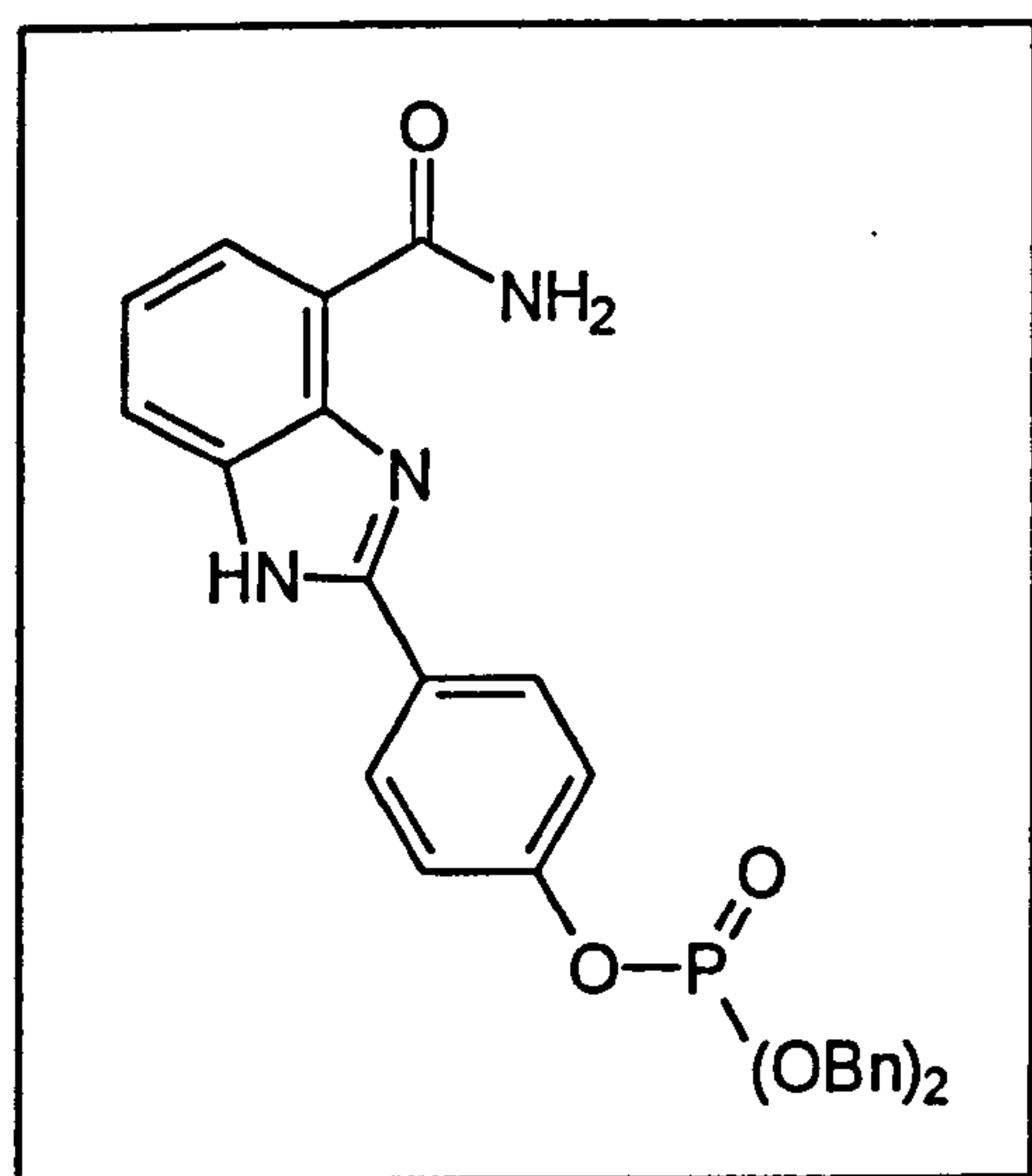


4-Carboxybenzaldehyde **154** (1.5 g, 0.01 M) was dissolved in dry THF (40 ml). *N,N*-Diisopropyl-*O*-benzyl isourea **155** (2.3 g, 0.01 M) was added and the reaction mixture was stirred at room temperature for 16 hours. As TLC indicated the presence of starting material, a further 0.47 g, 2 mmol of *N,N*-Diisopropyl-*O*-benzyl isourea **155** was added and the

reaction mixture was stirred for 12 hours. The reaction mixture was filtered through a small pad of silica which was washed with THF. The solvent was removed under reduced pressure to give an 'oily' solid, which was purified by column chromatography, using EtOAc:petrol 40/60 (2:8) as eluent to give a white solid (1.4 g, 6 mmol, 58%), m.p. 37-39 °C.

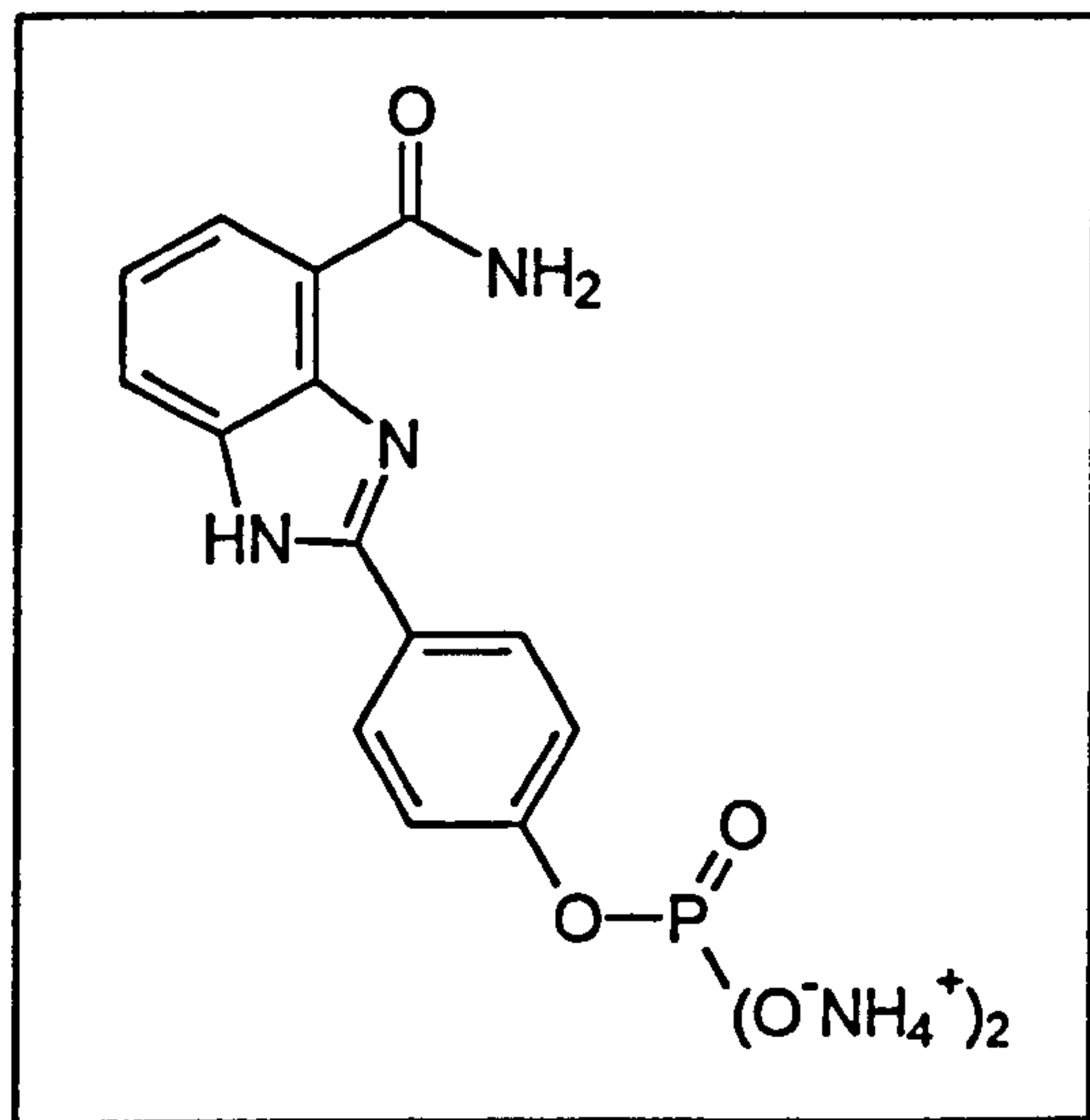
Anal. found C 75.07, H 5.00, $C_{15}H_{12}O_3$ requires C 74.99, H 5.03%; $\nu_{\max}/\text{cm}^{-1}$ 1723 and 1706 (C=O); δ_H (200 MHz, d_6 -DMSO) 5.49 (s, 2H, $\text{CH}_2\text{-Ph}$), 7.43-7.61 (m, 5H, Ph-2'/3'/4'/5'/6'H), 8.12-8.16 (d, 2H, Ar-2/6H, $J = 8.2$), 8.26-8.30 (d, 2H, Ar-3/5H, $J = 8.2$), 10.2 (s, 1H, CHO); m/z (EI) 240 (M^+ , 66%), 133 ($[M-\text{OBn}]^+$), 91 (Bn^+), 77 (Ph^+).

157 2-(4'-O-Dibenzylphosphorylphenyl)-1H-benzimidazole-4-carboxamide²⁸⁴



Prepared according to Method 2 for compound 98, from 2-(4'-hydroxyphenyl)-1H-benzimidazole-4-carboxamide 109 (0.5 g, 2.0 mmol). The product was purified by column chromatography using DCM:MeOH (9:1) as eluent, followed by recrystallisation from methanol/water to give pale yellow crystals (0.62 g, 1.2 mmol, 61%), m.p. 178-180 °C (lit. m.p.²⁵¹ 175-177 °C). Anal. found C 65.63, H 4.61, N 8.18, $C_{28}H_{24}N_3O_3P$ requires C 65.49, H 4.71, N 8.18%; $\nu_{\max}/\text{cm}^{-1}$ 3309 and

3226 (1° amide NH), 1666 and 1609 (C=O), 1248 (P-O-aryl); δ_H (200 MHz, d_6 -DMSO) 5.29 (s, 2H, $-\text{CH}_2\text{Ph}$), 5.36 (s, 2H, $-\text{CH}_2\text{Ph}$), 7.45-7.52 (m, 3H, $2 \times \text{Bn-2''/3''/4''/5''/6''H}$, Ar-5H, Ph-3'/5'H), 7.83-7.86 (d, 1H, Ar-4H, $J = 7.9$), 7.93 (s, 1H, -NH), 7.96-8.00 (d, 1H, Ar-6H, $J = 6.7$), 8.33-8.38 (d, 2H, Ph-2'/6'H, $J = 8.6$); m/z (EI) 513 (M^+ , 2%), 300 ($[M-2 \times \text{OBn}]^+$), 253 ($[M-\text{P}(\text{O})(\text{OBn})_2\text{OH}]^+$), 91 (Bn^+).

159 2-(4'-O-Phosphorylphenyl)-1H-benzimidazole-4-carboxamide ammonium salt²⁸¹

Prepared in a similar way to compound 96, from 2-(4'-O-dibenzylphosphorylphenyl)-1H-benzimidazole-4-carboxamide **157** (0.2 g, 0.39 mmol). The crude product was washed through Celite using concentrated ammonia solution. The solvent was removed under reduced pressure to give an off-white product (0.2 g, 0.4 mmol, 74%), m.p. >370 °C.

δ_{H} (200 MHz, d_6 -DMSO) 7.32-7.49 (m, 3H, Ar-5H, Ar-4H), 7.82-7.92 (m, 3H, -NH, Ar-6H, Ph-2'/6'H), 8.25-8.29 (d, 2H, Ph-3'/5'H), 9.5 (s, 1H, -NH).

Chapter Nine: References

- 1 Friedberg EC (1995). Out of the shadows and into the light: the emergence of DNA repair. *TIBS*, 20, 381.
- 2 Collins ARS and Johnson RT (1984). The inhibition of DNA repair. *Adv. Rad. Biol.*, 11, 71-129.
- 3 Harris AL (1985). DNA repair: relationship to drug and radiation resistance, metastasis and growth factors. *Int. J. Radiat. Biol.*, 48, 675-690.
- 4 Lindahl T (1982). DNA repair enzymes. *Ann. Rev. Biochem.*, 51, 61-87.
- 5 Seeberg E, Eide L, Bjørås M (1995). The base excision repair pathway. *TIBS*, 20, 391-397.
- 6 Lehmann AR (1995). Nucleotide excision repair and the link with transcription. *TIBS*, 20, 402-405.
- 7 Doetsch PW (1995). What's old is new: an alternative DNA excision repair pathway. *TIBS*, 20, 384-386.
- 8 Friedberg EC, Walker GC and Seide W (1995). *DNA Repair*. ASM Press.
- 9 Kolodner RD (1995). Mismatch repair: mechanisms and relationship to cancer susceptibility. *TIBS*, 20, 387-401.
- 10 Newbold RF, Warren W, Medcalf ASC and Amos J (1980). Mutagenicity of carcinogenic methylating agents is associated with a specific DNA modification. *Nature*, 283, 596-599.
- 11 Voet D and Voet JG (1990). Section 31-5, Repair of DNA, 968, John Wiley & Sons, Inc.
- 12 Rydberg B, Lindahl T (1982). Nonenzymatic methylation of DNA by the intracellular methyl group donor S-adenosyl-L-methionine is a potentially mutagenic reaction. *EMBO J.*, 1, 211-216.
- 13 Swann PF, Waters TR, Moulton DC, Xu Y-Z, Zheng Q, Edwards M, Mace R (1996). Role of postreplicative DNA mismatch repair in the cytotoxic action of thioguanine. *Science*, 273, 1109-1111.
- 14 Karran P, Bignami M (1996). Drug-related killings: a case of mistaken identity. *Chem. & Biol.*, 3, 875-879.

- 15 Aquilina G, Giammarioli AM, Zijno A, Dimuccio A, Dogliotti E, Bignami M (1990). Tolerance to O⁶-methylguanine and 6-thioguanine cytotoxic effects: a cross-resistant phenotype in N-methylnitrosourea-resistant Chinese hamster ovary cells. *Cancer Res.*, **50**, 4248-4253.
- 16 Young RC (1990). Mechanisms to improve chemotherapy effectiveness. *Cancer*, **65**, 815-822.
- 17 Hayes JD and Wolf CR (1990). Molecular mechanisms of drug resistance. *Biochem. J.*, **272**, 281-295.
- 18 Perez RP, Hamilton TC, Ozols RF, Young RC (1993). Mechanisms and modulation of resistance to chemotherapy in ovarian cancer. *Cancer*, **71**, 1571-1580.
- 19 Bradley G, Ling V (1994). P-glycoprotein, multidrug resistance and tumour progression. *Cancer Metastasis Rev.*, **13**, 223-233.
- 20 Bonadonna G, Valagussa P, Tancini G (1980). Estrogen-receptor status and response to chemotherapy in early and advanced breast cancer. *Cancer Chemother. Pharmacol.*, **4**, 37-41.
- 21 Hakala MT (1973). Enzyme changes in resistant tissues. In : *Drug Resistance and Selectivity : Biochemical and Cellular Basis* (E. Mihich, ed.), 264-298, Academic Press, New York, London.
- 22 Kaina B, Fritz G, Mitra S and Coquerelle T (1991). Transfection and expression of human O⁶-methylguanine-DNA methyltransferase (MGMT) cDNA in Chinese hamster cells: the role of MGMT in protection against the genotoxic effects of alkylating agents. *Carcinogenesis*, **12**, 1857-1867.
- 23 Hill AT (1996). Drug resistance: An overview of the current state of the art. *Int. J. Oncol.*, **9**, 197-203.
- 24 Skovsgaard T, Nielson D, Maare C and Wasserman K (1994). Cellular resistance to cancer chemotherapy. *Int. Rev. Cytol.*, **156**, 77-157.
- 25 Hu XF, Slater A, Wall DM, Kantharidis P, Parkin JD, Cowman A, Zalcberg JR (1995). Rapid up-regulation of *mdr1* expression by anthracyclines in a classical multidrug-resistant cell line. *Br. J. Cancer*, **71**, 931-936.

- 26 Yang X, Page M (1995). P-glycoprotein expression in ovarian cancer cell lines following treatment with cisplatin. *Oncol. Res.*, 7, 619-624.
- 27 Slovak ML, Ho JP, Bhardwaj G, Kurz EU, Deeley RG, Cole SPC (1993). Localization of a novel multidrug-resistance-associated gene in the HT1080/DR4 and H69AR human tumor-cell lines. *Cancer Res.*, 53, 3221-3225.
- 28 Lane M (1973). Clinical resistance to cancer chemotherapy. In: *Drug Resistance and Selectivity: Biochemical and Cellular Basis* (E. Mihich, ed.), 453-479, Academic Press, New York, London.
- 29 Ueda K, Hayaishi O (1985). ADP-Ribosylation. *Ann. Rev. Biochem.*, 54, 73-100.
- 30 Chambon P, Weil JD, Mandel P (1963). Nicotinamide mononucleotide activation of a new DNA-dependent polyadenylic acid synthesising nuclear enzyme. *Biochem. Biophys. Res. Commun.*, 11, 39-43.
- 31 Chambon P, Weil JD, Doly J, Strosser MT, Mandel P (1966). On the formation of a novel adenylic compound by enzymatic extracts of liver nuclei. *Biochem. Biophys. Res. Commun.*, 25, 638-643.
- 32 Collier RJ, Pappenheimer AM Jr. (1964). Studies on the mode of action of diphtheria toxin II. Effect of toxin on amino acid incorporation in cell-free systems. *J. Exp. Med.*, 120, 1019-1039.
- 33 De Murcia G, Niedergang C, Bürkle A (1995). Proceedings of the 11th International Symposium on ADP-ribosylation - Introduction. *Biochimie*, 77, 311.
- 34 Clapper DL, Walseth TF, Dargie PJ, Lee HC (1987). Pyridine nucleotide metabolites stimulate calcium release from sea urchin egg microsomes desensitized to inositol trisphosphate. *J. Biol. Chem.*, 262, 9561-9568.
- 35 Lee HC, Walseth TF, Bratt GT, Hayes RN, Clapper DL (1989). Structural determination of a cyclic metabolite of NAD⁺. *J. Biol. Chem.*, 264, 9561-9568.
- 36 Okazaki IJ, Moss L (1994). Common structure of the catalytic sites of mammalian and bacterial toxin ADP-ribosyltransferases. *Mol. Cell. Biochem.*, 138, 177-181.
- 37 Shall S (1995). ADP-ribosylation reactions. *Biochimie*, 77, 313-318.
- 38 Lee HC (1994). Cyclic ADP-ribose: A calcium mobilizing metabolite of NAD⁺. *Mol. Cell. Biochem.*, 138, 229-235.

- 39 Takasawa S, Nata K, Yonekura H, Okamoto H (1993). Cyclic ADP-ribose in insulin secretion from pancreatic β -cells. *Science*, 259, 370-373.
- 40 Walseth TF, Aarhus R, Zeleznikar RJ Jr., Lee HC (1991). Determination of endogenous levels of cyclic ADP-ribose in rat tissues. *Biochem. Biophys. Acta*, 1094, 113-120.
- 41 Lee HC, Aarhus R, Levitt D (1994). The crystal structure of cyclic ADP-ribose. *Nature Struct. Biol.*, 1, 143-144.
- 42 Jacobson MK, Cervantes-Laurean D, Strohm MS, Coyle DL, Bummer PM, Jacobson EL (1995). NAD glycohydrolases and the metabolism of cyclic ADP-ribose. *Biochimie*, 77, 341-344.
- 43 Lee HC, Graeff R, Walseth TF (1995). Cyclic ADP-ribose and its metabolic enzymes. *Biochimie*, 77, 345-355.
- 44 Takasawa S, Tohgo A, Noguchi N, Koguma T, Nata K, Sugimoto T, Yonekura H, Okamoto H (1993). Synthesis and hydrolysis of cyclic ADP-ribose by human leukocyte antigen CD38 and inhibition of the hydrolysis by ATP. *J. Biol. Chem.*, 268, 26052-26054.
- 45 Koguma T, Takasawa S, Tohgo A, Karasawa T, Furuya Y, Yonekura H, Okamoto H (1994). Cloning and characterisation of cDNA encoding rat ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase (homologue to human CD38) from islets of Langerhans. *Biochim. Biophys. Acta*, 1223, 160-162.
- 46 Howard M, Grimaldi JG, Bazan JF, Lund FE, Santos-Argumedo L, Parkhouse RME, Walseth TF, Lee HC (1993). Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. *Science*, 262, 1056-1059.
- 47 Schuber F, Travo P, Pascal M (1979). On the mechanism of action of calf spleen NAD^+ glycohydrolase. *Bioorg. Chem.*, 8, 83-90.
- 48 Tarnus C, Muller HM, Schuber F (1988). Chemical evidence in favor of a stabilised oxocarbenium-ion intermediate in the NAD^+ glycohydrolase-catalyzed reaction. *Bioorg. Chem.*, 16, 38-51.
- 49 Shall S (1984). ADP-Ribose in DNA repair: A new component of DNA excision repair. *Adv. Rad. Biol.*, 11, 1-69.

- 50 Boulikas Y (1991). Relation between carcinogenesis, chromatin structure and poly ADP-ribosylation. *Anticancer Res.*, 11, 489-528.
- 51 Hayashi K, Tanaka M, Shimada T, Miwa M, Shimura T (1983). Size and shape of poly(ADP-ribose): Examination by gel filtration, gel electrophoresis and electron microscopy. *Biochem. Biophys. Res. Commun.*, 112, 102-107.
- 52 Burtscher HJ, Auer B, Klocker H, Schweiger M, Hirsch-Kaufmann M (1986). Isolation of ADP-ribosyltransferase by affinity chromatography. *Anal. Biochem.*, 152, 285-290.
- 53 Ushiro H, Yokoyama Y, Shizuta Y (1987). Purification and characterization of poly(ADP-ribose) polymerase from human placenta. *J. Biol. Chem.*, 262, 2352-2357.
- 54 Mandel, P Okazaki H, Niedergang C (1977). Purification and properties of calf thymus poly adenosine diphosphate ribose polymerase. *FEBS Lett.*, 84, 331-336.
- 55 Burtscher HJ, Schneider R, Klocker H, Auer B, Hirsch-Kauffman M, Schweiger M (1987). ADP-ribosyltransferase is highly conserved: Purification and characterization of ADP-ribosyltransferase from a fish and its comparison with the human enzyme. *J. Comp. Physiol.*, 157, 567-572.
- 56 Burtscher HJ, Klocker H, Schneider R, Auer B. Hirsch-Kauffman M, Schweiger M (1987). ADP-ribosyltransferase from *Helix pomatia*: Purification and characterization. *Biochem. J.*, 248, 859-864.
- 57 Rickwood D, Osman MS (1979). Characterization of poly(ADP-ribose) polymerase activity in nuclei from the slime mould *Dictyostelium discoideum*. *Mol. Cell. Biochem.*, 23, 79-84.
- 58 Werner E, Sohst S, Gropp F, Simon D, Wagner H, Kröger H (1984). Presence of poly(ADP-ribose) polymerase and poly(ADP-ribose) glycohydrolase in the dinoflagellate *Cryptothecodinium cohnii*. *Eur. J. Biochem.*, 139, 81-86.
- 59 Uchida K, Miwa M (1994). Poly(ADP-ribose) polymerase: Structural conservation among different classes of animals and its implications. *Mol. Cell. Biochem.*, 138, 25-32.

- 60 Alkhatib HM, Chen D, Cherney B, Bhatia K, Notario V, Giri C, Stein G, Slattery E, Roeder RG, Smulson ME (1987). Cloning and expression of cDNA for human poly(ADP-ribose) polymerase. *Proc. Natl. Acad. Sci. USA*, 84, 1224-1228.
- 61 Suzuki H, Uchida K, Shima H, Sato T, Okamoto T, Kimura T, Miwa M (1987). Molecular cloning of cDNA for human poly(ADP-ribose) polymerase and expression of its gene during HL-60 cell differentiation. *Biochem. Biophys. Res. Commun.*, 146, 403-409.
- 62 Huppi K, Bhatia K, Siwarski D, Klinman D, Cherney B, Smulson ME (1989). Sequence and organisation of the mouse poly(ADP-ribose) polymerase gene. *Nucleic Acids Res.*, 17, 3387-3401.
- 63 Thibodeau J, Gradwohl G, Dumas C, Clairoux-Moreau S, Brunet G, Penning C, Poirier GG, Morea P (1989). Cloning of rodent cDNA encoding the poly(ADP-ribose) polymerase catalytic domain and analysis of mRNA levels during the cell cycle. *Biochem. Cell. Biol.*, 67, 653-660.
- 64 Saito I, Hatakeyama K, Kido TG, Ohkubo H, Nakanishi S, Ueda K (1990). Cloning of a full length cDNA encoding bovine thymus poly(ADP-ribose) synthetase: evolutionarily conserved segments and their potential functions. *Gene*, 90, 249-254.
- 65 Ittel M-E, Garnier J-M, Jeltsh J-M, Niedergang CP (1991). Chicken poly(ADP-ribose) synthetase: complete deduced amino acid sequence and comparison with mammalian enzyme sequences. *Gene*, 102, 157-164.
- 66 Ozawa Y, Uchida K, Uchida M, Ami Y, Kushida S, Okada N, Miwa M (1993). Isolation of cDNAs encoding the catalytic domain of poly(ADP-ribose) polymerase from *Xenopus laevis* and cherry salmon using heterologous oligonucleotide consensus sequences. *Biochem. Biophys. Res. Commun.*, 193, 119-125.
- 67 Uchida K, Hanai S, Ishikawa K, Ozawa Y, Uchida M, Sugimura T, Miwa M (1993). Cloning of cDNA encoding *Drosophila* poly (ADP-ribose) polymerase: leucine zipper in the automodification domain. *Proc. Natl. Acad. Sci. USA*, 90, 3481-3485.

- 68 De Murcia G, Schreiber V, Molinete M, Saulier B, Poch O, Masson M, Niedergang C, Ménissier de Murcia J (1994). Structure and function of poly(ADP-ribose) polymerase. *Mol. Cell. Biochem.*, 138, 15-24.
- 69 De Murcia G, Ménissier de Murcia J (1994). Poly (ADP-ribose) polymerase : a molecular nick-sensor. *TIBS*, 19, 172-176.
- 70 Lepiniec L, Babiychuk E, Kushnir S, Van Montagu M, Inzé D (1995). Characterization of an *Arabidopsis thaliana* cDNA homologue to animal poly(ADP-ribose) polymerase. *FEBS Lett.*, 364, 103-108.
- 71 Lin YH (1976). Detection and possible function of NAD⁺-incorporating activity in various plants. *Proc. Natl. Acad. Sci. USA*, 9, 21-28.
- 72 Payne JF, Bal AK (1976). Cytological detection of poly(ADP-ribose) polymerase. *Exp. Cell. Res.*, 99, 428-432.
- 73 Uchida KT, Morita T, Sato T, Ogura T, Yamashita R, Noguchi S, Suzuki H, Nyunoya H, Miwa M Sugimura T (1987). Nucleotide sequence of a full-length cDNA for human fibroblast poly (ADP-ribose) polymerase. *Biochem. Biophys. Res. Commun.*, 148, 617-622.
- 74 Benjamin RC, Gill DM (1980). Poly(ADP-ribose) synthesis *in vitro* programmed by damaged DNA. A comparison of DNA molecules containing different types of strand breaks. *J. Biol. Chem.*, 255, 10501-10508.
- 75 Zahradka P, Ebisuzaki K (1984). Poly(ADP-ribose) polymerase is a zinc metallo-enzyme. *Eur. J. Biochem.*, 142, 503-509.
- 76 Nishikimi N, Ogasawara K, Kameshita I, Taniguchi T, Shizuta Y (1982). Poly(ADP-ribose) synthetase. The DNA binding domain and the automodification domain. *J. Biol. Chem.*, 257, 6102-6105.
- 77 Kameshita I, Matsuda Z, Taniguchi T, Shizuta Y (1984). Poly(ADP-ribose) synthetase. Separation and identification of the three proteolytic fragments as the substrate binding domain, the DNA-binding domain, and the automodification domain. *J. Biol. Chem.*, 259, 4770-4776.
- 78 Mazen A, Ménissier de Murcia J, Molinete M, Simonin F, Gradwohl G, Poirier GG, de Murcia G (1989). Poly(ADP-ribose) polymerase: A novel finger protein. *Nucleic Acids Res.*, 17, 4689-4698.

- 79 Trapp CL, Simbulan-Rosenthal C, Smulson ME, Keen CL (1997). Effect of zinc deficiency on poly(ADP-ribose) polymerase in 3T3 cells. *FASEB J.*, 11, 1118 (meeting abstract).
- 80 Muller S, Briand J-P, Barakat S, Lagueux J, Poirier GG, de Murcia G, Isenberg DA (1994). Autoantibodies reacting with poly(ADP-ribose) and with a zinc finger functional domain of poly(ADP-ribose) polymerase involved in the recognition of damaged DNA. *Clin. Immunol. Immunopath.*, 73, 187-196.
- 81 Sugimura T, Miwa M (1994). Poly(ADP-ribose): Historical perspective. *Mol. Cell. Biochem.*, 138, 5-12.
- 82 Ikejima M, Noguchi S, Yamashita R, Ogura T, Sugimura T, Gill DM, Miwa M (1990). The zinc fingers of human poly(ADP-ribose) polymerase are differentially required for the recognition of DNA breaks and nicks, and the consequent enzyme activation: Other structures recognise intact DNA. *J. Biol. Chem.*, 265, 21907-21913.
- 83 Gradwohl G, Ménissier de Murcia J, Molinete M, Simonin F, Koken M, Hoeijmakers JHJ, de Murcia G (1990). The second zinc-finger domain of poly(ADP-ribose) polymerase determines specificity for single-stranded breaks in DNA. *Proc. Natl. Acad. Sci. USA*, 87, 2990-2994.
- 84 Mazen A, Gradwohl G, de Murcia G (1988). Zinc-binding protein detected by protein blotting. *Anal. Biochem.*, 172, 39-42.
- 85 Schreiber V, Molinete M, Boeuf H, de Murcia G, Ménissier de Murcia J (1992). The human poly(ADP-ribose) polymerase localization signal is a bipartite element functionally separate from DNA binding and catalytic activity. *EMBO J.*, 11, 3263-3269.
- 86 Buki K, Kun E (1988). Polypeptide domains of ADP-ribosyltransferase obtained by digestion with plasmin. *Biochemistry*, 27, 5990-5995.
- 87 Trucco C, Flatter E, Fribourg S, de Murcia G, Ménissier de Murcia J (1996). Mutations in the amino-terminal domain of the human poly(ADP-ribose) polymerase that affect its catalytic activity but not its DNA binding capacity. *FEBS Lett.*, 399, 313-316.

- 88 Ménissier de Murcia J, Molinete M, Gradwohl G, Simonin F, de Murcia G (1989). Zinc-binding domain of poly(ADP-ribose) polymerase participates in the recognition of single strand breaks on DNA. *J. Mol. Biol.*, **210**, 229-233.
- 89 Le Cam E, Fack F, Ménissier de Murcia J, Cognet JAH, Barbin A, Sarantoglou V, Révet B, Delain E, de Murcia G (1994). Conformational analysis of a 139 base-pair DNA fragment containing a single-stranded break and its interaction with human poly(ADP-ribose) polymerase. *J. Mol. Biol.*, **235**, 1062-1071.
- 90 Yoshihara K, Hashida T, Tanaka Y, Ohgushi H (1971). Enzyme-bound early product of purified poly(ADP-ribose) polymerase. *Biochem. Biophys. Res. Commun.*, **78**, 1281-1288.
- 91 Kawaichi M, Ueda K, Hayaishi O (1981). Multiple auto-poly(ADP-ribosylation) of rat liver poly(ADP-ribose). synthetase: Mode of modification and properties of automodified synthetase. *J. Biol. Chem.*, **256**, 9483-9489.
- 92 Adamietz P (1987). Synthetase is the major endogenous non-histone acceptor for poly(ADP-ribose) in alkylated rat hepatoma cells. *Eur. J. Biochem.*, **169**, 365-372.
- 93 Desmarais Y, Ménard L, Lagueux J, Poirier GG (1991). Enzymological properties of poly(ADP-ribose) polymerase: characterization of automodification sites and NADase activity. *Biochim. Biophys. Acta*, **1078**, 179-186.
- 94 Kim H, Jacobson MK, Rolli V, Ménissier de Murcia J, Reinbolt J, Simonin F, Ruf A, Schulz G, de Murcia G (1997). Photoaffinity labeling of human poly(ADP-ribose) polymerase catalytic domain. *Biochem. J.*, **322**, 469-475.
- 95 Simonin F, Ménissier de Murcia J, Poch O, Muller S, Gradwohl G, Molinete M, Penning C, Keith G, de Murcia G (1990). Expression of site-directed mutagenesis of the catalytic domain of human poly(ADP-ribose) polymerase in *Escherichia coli*. *J. Biol. Chem.*, **265**, 19249-19256.
- 96 Simonin F, Höfferer L, Panzeter PL, Muller S, de Murcia G, Althaus FR (1993). The carboxyl-terminal domain of human poly(ADP-ribose) polymerase. *J. Biol. Chem.*, **268**, 13454-13461.
- 97 Alvarez-Gonzalez R, Pacheco-Rodriguez G, Mendoza-Alvarez H (1994). Enzymology of ADP-ribose polymer synthesis. *Mol. Cell. Biochem.*, **138**, 33-37.

- 98 Kawaichi M, Ueda K, Hayaishi O (1980). Initiation of poly(ADP-ribosyl)-histone synthesis of poly(ADP-ribose) synthetase. *J. Biol. Chem.*, **255**, 816-819.
- 99 Althaus FR, Richter CR (1987). ADP-ribosylation of proteins. *Mol. Biol. Biochem. Biophys.*, **37**, 1-126.
- 100 Miwa M, Saikawa N, Yamaizumi Z, Nishimura S, Sugimura T (1979). Structure of poly(adenosine diphosphate ribose): Identification of 2'-(1''-ribosyl-2''-(or 3'')-(1''-ribosyl) adenosine-5',5'',5'''-tris(phosphate) as a branch linkage. *Proc. Natl. Acad. Sci. USA*, **76**, 595-599.
- 101 Slama JT, Simmons AM (1988). Carbanicotinamide adenine dinucleotide: synthesis and enzymological properties of a carbocyclic analogue of oxidized nicotinamide adenine dinucleotide. *Biochemistry*, **27**, 183-193.
- 102 Slama JT, Simmons AM (1989). Inhibition of NAD glycohydrolase and ADP-ribosyltransferase by carbocyclic analogues of oxidized NAD⁺. *Biochemistry*, **28**, 7688-7694.
- 103 Oppenheimer NJ (1994). NAD hydrolysis: Chemical and enzymatic mechanisms. *Mol. Cell. Biochem.*, **138**, 245-251.
- 104 Alvarez-Gonzalez R, Mendoza-Alvarez H (1993). Poly(ADP-ribose) Polymerase is a catalytic dimer and the automodification reaction is intermolecular. *J. Biol. Chem.*, **268**, 22575-22580.
- 105 Bauer PI, Buki KG, Hakam A, Kun E (1990). Macromolecular association of ADP-ribosyltransferase and its correlation with enzyme activity. *Biochem. J.*, **270**, 17-26.
- 106 Panzeter P, Althaus FR (1994). DNA strand break-mediated partitioning of poly(ADP-ribose) polymerase function. *Biochemistry*, **33**, 9600-9605.
- 107 Alvarez-Gonzalez R, Mendoza-Alvarez H (1995). Dissection of ADP-ribose polymer synthesis into individual steps of initiation, elongation and branching. *Biochimie*, **77**, 403-407.
- 108 Baker PJ, Britton KL, Engel PC, Farrants GW, Lilley KS, Rice DW, Stillman TJ (1992). Subunit assembly and active site location in the structure of glutamate dehydrogenase. *Proteins*, **12**, 75-86.

- 109 Rossman MG, Moras K, Olsen KW (1974). Chemical and biological evolution of a nucleotide-binding protein. *Nature*, 250, 194-199.
- 110 Marsischky GT, Wilson BA, Collier RJ (1995). Role of glutamic acid 988 of human poly(ADP-ribose) polymerase in polymer formation: Evidence for active site similarities to the ADP-ribosylating toxins. *J. Biol. Chem.*, 270, 3247-3254.
- 111 Aktories K, Jung M, Böhmer J, Fritz G, Vandekerckhovec J, Just I (1995). Studies on the active-site structure of C3-like exoenzymes: Involvement of glutamic acid in catalysis of ADP-ribosylation. *Biochimie*, 77, 326-332.
- 112 Kofler B, Wallraff E, Herzog H, Schneider R, Auer B, Schweiger M (1993). Purification and characterization of NAD⁺:ADP-ribosyltransferase (polymerizing) from *Dictyostelium discoideum*. *Biochem. J.*, 293, 275-281.
- 113 Ueda K, Kawaichi M, Okayama H, Hayaishi O (1979). Poly(ADP-ribosyl).ation of nuclear proteins: Enzymatic elongation of chemically synthesised ADP-ribose histone adducts. *J. Biol. Chem.*, 254, 679-687.
- 114 Taniguchi T (1987). Reaction mechanism for automodification of poly(ADP-ribose) synthetase. *Biochem. Biophys. Res. Commun.*, 147, 1008-1012.
- 115 Alvarez-Gonzalez (1988). 3'-deoxy-NAD⁺ as a substrate for poly(ADP-ribose). polymerase and the reaction mechanism of poly(ADP-ribose) elongation. *J. Biol. Chem.*, 263, 17690-17696.
- 116 Alvarez-Gonzalez R, Jacobson MK (1987). Characterization of polymers of adenosine diphosphate ribose generated *in vitro* and *in vivo*. *Biochemistry*, 26, 3218-3224.
- 117 De Murcia G, Jongstra-Bilen J, Ittel M-E, Mandel P, Delain E (1983). Poly(ADP-ribose) polymerase automodification and interaction with DNA. *EMBO J.*, 2, 543-548.
- 118 Miwa M, Sugimura T (1971). Splitting of the ribose-ribose linkage of poly(adenosine diphosphate-ribose) by a calf thymus extract. *J. Biol. Chem.*, 246, 6362-6364.
- 119 Ueda K, Okea J, Narumiya S, Miyakawa N, Hayaishi O (1972). Poly ADP ribose glycohydrolase from rat liver nuclei, a novel enzyme degrading the polymer. *Biochem. Biophys. Res. Commun.*, 46, 516-523.

- 120 Miwa M, Tanaka M, Matsushima T, Sugimura T (1974). Purification and properties of a glycohydrolase from calf thymus splitting ribose-ribose linkages of poly(adenosine diphosphate-ribose). *J. Biol. Chem.*, **249**, 3475-3482.
- 121 Lautier D, Lagueux J, Thibodeau J, Ménard L, Poirier GG (1993). Molecular and biochemical features of poly(ADP-ribose) metabolism. *Mol. Cell. Biochem.*, **122**, 171-193.
- 122 Brochu G, Shah GM, Poirier GG (1994). Purification of poly(ADP-ribose) glycohydrolase and detection of its isoforms by a zymogram following one- or two-dimensional electrophoresis. *Anal. Biochem.*, **218**, 265-272.
- 123 Tanuma S, Endo H (1990). Purification and characterization of an (ADP-ribose)_n glycohydrolase from human erythrocytes. *Eur. J. Biochem.*, **191**, 57-63.
- 124 Hatakeyama K, Nemoto Y, Ueda K, Hayaishi O (1986). Purification and characterization of poly(ADP-ribose) glycohydrolase. Different modes of action on large and small poly(ADP-ribose). *J. Biol. Chem.*, **261**, 14902-14911.
- 125 Jonsson GG, Jacobson EL, Jacobson MK (1988). Mechanism of alteration of poly(adenosine diphosphate-ribose) metabolism by hyperthermia. *Cancer Res.*, **48**, 4233-4239.
- 126 Alvarez-Gonzalez R, Althaus FR (1989). Poly(ADP-ribose) catabolism in mammalian cells exposed to DNA-damaging agents. *Mutat. Res.*, **218**, 67-74.
- 127 Ikejima M, Gill DM (1988). Poly(ADP-ribose) degradation by glycohydrolase starts with an endonucleolytic incision. *J. Biol. Chem.*, **263**, 11037-11040.
- 128 Brochu G, Duchaine C, Thibeault L, Lagueux J, Shah GM, Poirier GG (1994). Mode of action of poly(ADP-ribose) glycohydrolase. *Biochim. Biophys. Acta*, **1219**, 342-350.
- 129 Desnoyers S, Shah GM, Brochu G, Hoflack JC, Verreault A, Poirier GG (1995). Biochemical properties and function of poly(ADP-ribose) glycohydrolase. *Biochimie*, **77**, 433-438.
- 130 Althaus FR (1992). Poly ADP-ribosylation: a histone shuttle mechanism in DNA excision repair. *J. Cell. Sci.*, **102**, 663-670.

- 131 Okayama H, Honda M, Hayaishi O (1978). Novel enzyme from rat liver that cleaves an ADP-ribosyl histone linkage. *Proc. Natl. Acad. Sci. USA*, **75**, 2254-2257.
- 132 Oka J, Ueda K, Hayaishi O, Komura H, Nakanishi K (1984). ADP-ribosyl protein lyase. Purification, properties and identification of the product. *J. Biol. Chem.*, **259**, 986-993.
- 133 Ludwig A, Behnke B, Holtlund J, Hiltz H (1988). Immunoquantitation and size determination of intrinsic poly(ADP-ribose) polymerase from acid precipitates. An analysis of the *in vivo* status in mammalian species and in lower eukaryotes. *J. Biol. Chem.*, **263**, 6993-6999.
- 134 Yamanaka H, Penning CA, Willis EH, Wasson DB, Carson DA (1988). Characterization of human poly(ADP-ribose) polymerase with autoantibodies. *J. Biol. Chem.*, **263**, 3879-3883.
- 135 Tanuma S, Kawashima K, Endo H (1986). Acceptor proteins for poly(ADP-ribose)_n in the HeLa S3 cell cycle. *J. Biol. Chem. (Tokyo)*, **99**, 915-922.
- 136 De Murcia G, Huletsky A, Poirier GG (1988). Modulation of chromatin structure by poly(ADP-ribosylation). *Biochem. Cell. Biol.*, **66**, 626-635.
- 137 Kaufmann SH, Brunet G, Talbot B, Lamarr D, Dumas C, Shaper JH, Poirier GG (1991). Association of poly(ADP-ribose) polymerase with the nuclear matrix: The role of intermolecular disulfide bond formation, RNA retention and cell type. *Exp. Cell. Res.*, **192**, 524-535.
- 138 Roberts JH, Stark O, Giri CP, Smulson M (1975). Cytoplasmic poly(ADP-ribose) polymerase during the HeLa cell cycle. *Arch. Biochem. Biophys.*, **171**, 305-315.
- 139 Jesser M, Chypre C, Hog F, Mandel P (1993). Cytoplasmic poly(ADP-ribose) polymerase from mouse messenger ribonucleoprotein particles: Purification and characterization. *Biochim. Biophys. Res. Commun.*, **195**, 558-564.
- 140 Desnoyers S, Kaufmann SH, Poirier GG (1996). Alteration of the nuclear localization of poly(ADP-ribose) polymerase upon treatment with transcription inhibitors. *Exp. Cell. Res.*, **227**, 146-153.
- 141 Boulikas T (1992). Poly(ADP-ribose) synthesis in blocked and damaged cells and its relation to carcinogenesis. *Anticancer Res.*, **12**, 885-898.

- 142 Hunting DJ, Gowans BJ, Henderson JF (1985). Specificity of inhibitors of poly(ADP-ribose) synthesis. Effects on nucleotide metabolism in cultured cells. *Mol. Pharmacol.*, **28**, 200-206.
- 143 Milam KM, Thomas GH, Cleaver JE (1986). Disturbances in DNA precursor metabolism associated with exposure to an inhibitor of poly(ADP-ribose) synthetase. *Exp. Cell Res.*, **165**, 260-268.
- 144 De Murcia G, Masson M, Trucco C, Dantzer F, Oliver J, Flatter E, Niedergang C, Ricoul M, Dutrillaux B, Dierich A, LeMeur M, Waltzinger C, Chambon P, Ménissier de Murcia J (1997). Poly(ADP-ribose) polymerase interacts with base excision repair enzymes and is required in recovery from DNA damage in mice and cells. *Proceedings of the 12th International Symposium on ADP-Ribosylation Reactions (meeting abstract)*, Cancun, Mexico, May 10-14th.
- 145 Wang Z, Auer B, Stingl L, Berghammer H, Haidacher D, Schweiger M, Wagner E (1995). Mice lacking in ADPRT and poly(ADP-ribosyl)ation develop normally but are susceptible to skin disease. *Genes Devel.*, **9**, 509-520.
- 146 Ueda K, Reeder RH, Honjo T, Nishizuka Y, Hayaishi O (1968). Poly adenosine diphosphate ribose synthesis associated with chromatin. *Biochem. Biophys. Res. Commun.*, **31**, 379-385.
- 147 Poirier GG, de Murcia G, Jongstra-Bilen J, Niedergang C, Mandel P (1982). Poly(ADP-ribosyl)ation of polynucleosomes causes relaxation of chromatin structure. *Proc. Natl. Acad. Sci. USA*, **20**, 3423-3427.
- 148 De Murcia G, Huletsky A, Lamarre D, Gaudreau A, Pouyet J, Poirier GG (1985). Poly (ADP-ribose) glycohydrolase activity causes recondensation of relaxed poly(ADP-ribosyl)ated polynucleosomes. In *ADP-ribosylation of proteins*. (Althaus FR, Hilz H, Shall S, eds.), Springer-Verlag, Berlin, Heidelberg, 190-196.
- 149 De Murcia G, Huletsky A, Lamarre D, Gaudreau A, Pouyet J, Daune M, Poirier GG (1986). Modulation of chromatin superstructure induced by poly(ADP-ribose) synthesis and degradation. *J. Biol. Chem.*, **261**, 7011-7017.
- 150 Niedergang C, de Murcia G, Ittel ME, Pouyet J, Mandel P (1985). Time course of polynucleosome relaxation and ADP-ribosylation. Correlation between relaxation and histone H1 hyper-ADP-ribosylation. *Eur. J. Biochem.*, **146**, 185-191.

- 151 Butt TR, Decoste B, Jump DB, Nolan N, Smulson M (1980). Characterization of a putative poly(adenosine diphosphate ribose)-chromatin complex. *Biochemistry*, 19, 5243-5249.
- 152 Cleaver JE, Morgan WF (1991). Poly(ADP-ribose) polymerase: a perplexing participant in cellular responses to DNA breakage. *Mutat. Res.*, 257, 1-18.
- 153 Ogata N, Ueda K, Kawaichi M, Hayaishi O (1981). Poly(ADP-ribose) synthetase, a main acceptor of poly(ADP-ribose) in isolated nuclei. *J. Biol. Chem.*, 256, 4135-4137.
- 154 Yoshihara K, Tanigawa Y, Burzio L, Koide SS (1975). Evidence for adenosine diphosphate ribosylation of Ca^{2+} , Mg^{2+} dependent endonuclease. *Proc. Natl. Acad. Sci. USA*, 72, 289-293.
- 155 Yoshihara K, Itaya A, Tanaka Y, Ohashi Y, Ito K, Treaoka H, Tsukada K, Matsukage A, Kamiya T (1985). Inhibition of DNA polymerase α , DNA polymerase β , terminal nucleotidyl-transferase and DNA ligase II by poly(ADP-ribosyl)ation reaction in vitro. *Biochem. Biophys. Res. Commun.*, 128, 61-67.
- 156 Ferro AM, Olivera BM (1984). Poly(ADP-ribosylation) of DNA topoisomerase I from calf thymus. *J. Biol. Chem.*, 259, 547-554.
- 157 Ferro AM, Thompson LH, Olivera BM (1984). Poly(ADP-ribosyl)ation and DNA topoisomerase I in different cell lines. *In* : Proteins Involved in DNA Replication (Hübscher U and Spadari S, eds.), Plenum, New York, 441-447.
- 158 Darby MK, Schmitt B, Jongstra-Bilen J, Vosberg HP (1985). Inhibition of calf thymus type II DNA topoisomerase by poly(ADP-ribosylation). *EMBO J.*, 4, 2129-2134.
- 159 Taniguchi T, Suzuki S, Shizuta Y (1985). Poly(ADP-ribosyl)ation of RNA polymerase II from wheat germ. *Biochem. Biophys. Res. Commun.*, 127, 526-532.
- 160 Suzuki H, Quesada P, Farina B, Leone E (1986). In vitro poly(ADP-ribosyl)ation of seminal ribonuclease. *J. Biol. Chem.*, 261, 6048-6055.
- 161 Hussani MZ, Ghani QP, Hunt TK (1989). Inhibition of prolyl hydroxylase by poly(ADP-ribose) and phosphoribosyl-AMP : Possible role of ADP-ribosylation in intracellular prolyl hydroxylase regulation. *J. Biol. Chem.*, 264, 7850-7855.

- 162 Tanuma S, Yagi T, Johnson GS (1985). Endogenous ADP-ribosylation of high mobility group proteins 1 and 2 and histone H1 following DNA damage in intact cells. *Arch. Biochem. Biophys.*, 237, 38-42.
- 163 Adamietz P, Rudolph A (1984). ADP-ribosylation of nuclear proteins in vivo. Identification of histone H2B as a major acceptor for mono- and poly(ADP-ribose) in dimethyl sulfate treated hepatoma AH7974 cells. *J. Biol. Chem.*, 259, 6841-6846.
- 164 Goldman N, Brown M, Khoury G (1981). Modification of SV40 T antigen by poly ADP-ribosylation. *Cell*, 24, 567-572.
- 165 Ohashi Y, Haya A, Tanaka Y, Yoshihara K, Kamiya T, Matsukage A (1986). Poly(ADP-ribosyl)ation of DNA polymerase β in vitro. *Biochem. Biophys. Res. Commun.*, 140, 666-673.
- 166 Wiencke JK, Morgan WF (1987). Cell cycle-dependent potentiation of X-ray-induced chromosomal aberrations by 3-aminobenzamide. *Biochem. Biophys. Res. Commun.*, 143, 372-376.
- 167 Oikawa A, Tohda H, Kanai M, Miwa M, Sugimura T (1980). Inhibitors of poly(adenosine diphosphate ribose) polymerase induce sister chromatid exchanges. *Biochem. Biophys. Res. Commun.*, 97, 1311-1316.
- 168 Hori T-A (1981). High incidence of sister chromatid exchanges and chromatid interchanges in the conditions of lowered activity of poly(ADP-ribose) polymerase. *Biochem. Biophys. Res. Commun.*, 102, 38-45.
- 169 Morgan WF, Cleaver JE (1982). 3-Aminobenzamide synergistically increases sister-chromatid exchanges in cells exposed to methyl methanesulfonate but not to ultraviolet light. *Mutat. Res.*, 104, 361-366.
- 170 Bürkle A, Heilbronn R, zur Hausen H (1990). Potentiation of carcinogen-induced methotrexate resistance and dihydrofolate reductase gene amplification by inhibitors of poly(adenosine diphosphate-ribose) polymerase. *Cancer Res.*, 50, 5756-5760.
- 171 Bürkle A, Meyer T, Hilz H, Zur HH (1987). Enhancement of N-methyl-N'-nitro-N-nitrosoguanidine-induced DNA amplification in a simian virus 40-transformed Chinese hamster cell-line by 3-aminobenzamide. *Cancer Res.*, 47, 3632-3636.

- 172 Ding R, Smulson M (1994). Depletion of nuclear poly(ADP-ribose) polymerase by antisense RNA expression: Influences on genomic stability, chromatin organization and carcinogen cytotoxicity. *Cancer Res.*, **54**, 4627-4634.
- 173 Durkacz BW, Omidiji O, Gray DA, Shall S (1980). (ADP-ribose)_n participates in DNA excision repair. *Nature*, **283**, 593-596.
- 174 Naegeli H, Loetscher P, Althaus FR (1989). Poly ADP-ribosylation of proteins. Processivity of posttranslational modification. *J. Biol. Chem.*, **264**, 14382-14385.
- 175 Naegeli H, Althaus (1991). Regulation of poly (ADP-ribose) polymerase. Histone-specific adaptations of reaction products. *J. Biol. Chem.*, **266**, 10596-10601.
- 176 Malanga M, Althaus FR (1994). Poly(ADP-ribose) molecules formed during DNA repair in vivo. *J. Biol. Chem.*, **269**, 17691-17696.
- 177 Malanga M, Althaus FR (1993). Effect of DNA damage on the numbers and size distributions of ADP-ribose polymers in vivo. *Experientia*, **49**, 2.
- 178 Panzeter P, Realini C, Althaus FR (1992). Noncovalent interactions of poly(adenosine diphosphate ribose) with histones. *Biochemistry*, **31**, 1379-1385.
- 179 Thoma F, Lose R, Koller (1983). Involvement of the domains of histones H1 and H5 in the structural organization of soluble chromatin. *J. Mol. Biol.*, **167**, 619-640.
- 180 Mathis G, Althaus (1987). Release of core DNA from nucleosomal core particles following (ADP-ribose)_n modification in vitro. *Biochem. Biophys. Res. Commun.*, **143**, 1049-1057.
- 181 Realini C, Althaus FR (1992). Histone shuttling by poly ADP-ribosylation. *J. Biol. Chem.*, **267**, 18858-18865.
- 182 Althaus FR, Höfferer L, Kleczkowska HE, Malanga M, Naegeli H, Panzeter PL, Realini CA (1994). Histone shuttling by poly(ADP-ribosyl)ation *Mol. Cell. Biochem.*, **138**, 53-59.
- 183 Althaus FR, Höfferer L, Kleczkowska HE, Malanga M, Naegeli H, Panzeter P, Realini C (1993). Histone shuttle driven by the automodification cycle of poly(ADP-ribose) polymerase. *Env. Mol. Mutag.*, **22**, 278-282.

- 184 Mathis G, Althaus FR (1990). Uncoupling of DNA excision repair and nucleosomal unfolding in poly(ADP-ribose)-depleted mammalian cells. *Carcinogenesis*, **11**, 1237-1239.
- 185 Satoh MS, Lindahl T (1992). Role of poly(ADP-ribose) formation in DNA repair. *Nature*, **356**, 356-358.
- 186 Chatterjee S, Berger NA (1994). Growth-phase-dependent response to DNA damage in poly(ADP-ribose) polymerase deficient cell-lines: basis for a new hypothesis describing the role of poly(ADP-ribose) polymerase in DNA replication and repair. *Mol. Cell. Biochem.*, **138**, 61-69.
- 187 Shall S (1994). The function of poly(ADP-ribosylation) in DNA breakage and rejoining. *Mol. Cell. Biochem.*, **138**, 71-75.
- 188 Durkacz BW, Irwin J, Shall S (1981). Inhibition of (ADP-ribose)_n biosynthesis retards DNA repair but does not inhibit DNA repair synthesis. *Biochem. Biophys. Res. Commun.*, **101**, 1433-1441.
- 189 James MR, Lehmann AR (1982). Role of poly(adenosine diphosphate ribose) in deoxyribonucleic acid repair in human fibroblasts. *Biochemistry*, **21**, 4007-4013.
- 190 Sims JL, Sikorski GW, Catino DM, Berger SJ, Berger NA (1982). Poly(adenosine-diphosphate ribose) polymerase inhibitors stimulate unscheduled deoxyribonucleic acid synthesis in normal human lymphocytes. *Biochemistry*, **21**, 1813-1821.
- 191 Creissen D, Shall S (1982). Regulation of DNA ligase activity by poly(ADP-ribose). *Nature (London)*, **296**, 271-272.
- 192 Cleaver JE, Morgan WF (1985). Poly (ADP-ribose) synthesis is involved in the toxic effects of alkylating agents but does not regulate DNA repair. *Mutat. Res.*, **150**, 69-76.
- 193 Küpper JH, de Murcia G, Bürkle A (1992). Inhibition of poly(ADP-ribosylation) by overexpressing the poly(ADP-ribose) polymerase DNA-binding domain. *J. Biol. Chem.*, **265**, 18721-18724.
- 194 Molinete M, Vermeulen W, Bürkle A, Ménissier de Murcia J, Küpper J, Hoeijmakers J, de Murcia G (1993). Overproduction of the poly(ADP-ribose)

- polymerase DNA-binding domain blocks alkylation-induced DNA repair synthesis in mammalian cells. *EMBO J.*, **12**, 2109-2117.
- 195 Küpper JH, van Gool L, Bürkle A (1995). Molecular genetic systems to study the role of poly(ADP-ribosyl)ation in the cellular response to DNA damage. *Biochimie*, **77**, 450-455.
 - 196 Eki T, Murakanmi Y, Enomoto T, Hanaoka F, Yamada M (1986). Characterization of DNA replication at a restrictive temperature in a mouse DNA temperature-sensitive mutant TSFT20 strain, containing heat-labile DNA polymerase- α activity. *J. Biol. Chem.*, **261**, 8888-8893.
 - 197 Eki T (1994). Poly(ADP-ribose) polymerase inhibits DNA replication by human replicative DNA polymerase α , δ and ϵ in vitro. *FEBS Lett.*, **356**, 261-266.
 - 198 Simbulan CMG, Suzuki M, Izuta S, Sakurai T, Savoysky E, Kojima K, Miyahara K, Shizuta Y, Yoshida S (1993). Poly(ADP-ribose) polymerase stimulates DNA polymerase α by physical association. *J. Biol. Chem.*, **268**, 93-99.
 - 199 Yoshida S, Simbulan CMG (1994). Interaction of poly(ADP-ribose) polymerase with DNA polymerase α . *Mol. Cell. Biochem.*, **138**, 39-44.
 - 200 Simbulan-Rosenthal CM, Rosenthal DS, Hilz H, Hickey R, Malkas L, Applegren N, Wu Y, Bers G, Smulson ME (1996). The expression of poly(ADP-ribose) polymerase during differentiation-linked DNA-replication reveals that it is a component of the multiprotein DNA-replication complex. *Biochemistry*, **35**, 11622-11633.
 - 201 White E (1996). Overview of Apoptosis. In Apoptosis : a complete catalog and scientific research guide, Oncogene Research Products, Calbiochem.
 - 202 Carson DA, Ribeiro JM (1993). Apoptosis and Disease. *Lancet*, **341**, 1251-1254.
 - 203 Thompson CB (1995). Apoptosis in the pathogenesis and treatment of disease. *Science*, **267**, 1456-1462.
 - 204 Korsmeyer SJ (1995). Regulators of cell death. *Trends Genet.*, **11**, 101-105.
 - 205 Hockenbery DM, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ (1990). Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature (London)*, **348**, 334-336.

- 206 Ottvai ZN, Milliman CL, Korsmeyer SJ (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, **74**, 609-619.
- 207 Chittenden T, Harrington EA, O'Connor R, Flemington C, Lutz RJ, Evan GI, Guild BC (1995). Induction of apoptosis by the Bcl-2 homologue Bak. *Nature (London)*, **374**, 733-736.
- 208 Martin SJ, Green DR (1995). Protease activation during apoptosis – death by 1000 cuts. *Cell*, **82**, 349-352.
- 209 Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS, Dixit VM (1995). Yama/CPP32 β , a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell*, **81**, 801-809.
- 210 Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG (1993). Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res.*, **53**, 3976-3985.
- 211 Adachi Y, Nishikimi A, Teraoka H, Ueda K (1997). Apoptotic degradation and subcellular localization of PARP regulated by phosphorylation. *Proceedings of the 12th International Symposium on ADP-Ribosylation Reactions (meeting abstract)*, Cancun, Mexico, May 10-14th.
- 212 Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC (1994). Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature (London)*, **371**, 346-347.
- 213 Shah GM, Shah RG, Poirier GG (1996). Different cleavage pattern for poly(ADP-ribose) polymerase during necrosis and apoptosis in HL-60 cells. *Biochem. Biophys. Res. Commun.*, **229**, 502-508.
- 214 Lautier D, Poirier D, Boudreau A, Alaoui-Jamali MA, Castonguay A, Poirier G (1990). Stimulation of poly(ADP-ribose) synthesis by free radicals in C3H10T1/2 cells: relationship with NAD metabolism and DNA breakage. *Biochem. Cell. Biol.*, **68**, 602-608.
- 215 Schranfstatter IU, Hinshaw DB, Hyslop PA, Spragg RG, Cochrane CG (1986). Oxidant injury of cells. DNA strand-breaks activate poly adenosine diphosphate

- ribose polymerase and lead to depletion of nicotinamide adenine dinucleotide. *J. Clin. Invest.*, **77**, 1312-1320.
- 216 Tanizawa A, Kubota M, Takimoto T, Akiyama T, Seto S, Kiriya Y, Mikawa H (1987). Prevention of adriamycin-induced interphase death by 3-aminobenzamide and nicotinamide in a human promyelocytic leukemia cell line. *Biochem. Biophys. Res. Commun.*, **144**, 1031-1036.
- 217 Ghibelli L, Nosseri C, Oliverio S, Piacentini M, Autuori F (1992). Cycloheximide can rescue heat-shocked L cells from death by blocking stress-induced apoptosis. *Exp. Cell. Res.*, **201**, 436-443.
- 218 Kuo M-L, Chau Y-P, Wang J-H, Shiah SG (1996). Inhibitors of poly(ADP-ribose) polymerase block nitric oxide-induced apoptosis but not differentiation in human leukaemia HL-60 cells. *Biochem. Biophys. Res. Commun.*, **219**, 502-508.
- 219 Hartwell LH, Weinert TA (1989). Checkpoints : controls that ensure the order of cell cycle events. *Science*, **246**, 629-634.
- 220 Hartwell LH (1992). Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell*, **71**, 543-546.
- 221 Nozaki T, Masutani M, Akagawa T, Sugimura T, Esumi H (1994). Suppression of G1 arrest and enhancement of G2 arrest by inhibitors of poly(ADP-ribose) polymerase : Possible involvement of poly(ADP-ribosylation) in cell cycle arrest following γ -irradiation. *Jpn. J. Cancer Res.*, **85**, 1094-1098.
- 222 Masutani M, Nozaki T, Wakabayashi K, Sugumura T (1995). Role of poly(ADP-ribose) polymerase in cell-cycle checkpoint mechanisms following γ -irradiation. *Biochimie*, **77**, 462-465.
- 223 Ameisen JC, Estaquier J, Idziorek T, De Bels F (1995). The relevance of apoptosis to AIDS pathogenesis. *Trends in Cell Biol.*, **5**, 27-32.
- 224 Tanaka Y, Yoshihara K, Kojima K, Itaya A, Kameoka M, Ikuto K, Kamiya T (1995). Poly(ADP-ribose) polymerase activity in various U937 cell subclones with different susceptibility to HIV-1 infection: its dramatic decrease following persistent virus infection. *Biochem. Biophys. Res. Commun.*, **213**, 161-168.
- 225 Allemand I, Angulo JF (1995). Transgenic and knockout models for studying DNA repair. *Biochimie*, **77**, 826-832.

- 226 Melton DW (1994). Gene targeting in the mouse. *Bioessays*, 16, 633-638.
- 227 Auer B, Flick K, Wang ZQ, Haidacher D, Jäger S, Berghammer H, Kofler B, Schweiger M, Wagner E (1995). On the biological role of the nuclear polymerizing NAD⁺: Protein (ADP-ribosyl) transferase (ADPRT): ADPRT from *Dictyostelium discoideum* and inactivation of the ADPRT gene in the mouse. *Biochimie*, 77, 444-449.
- 228 Satoh MS, Poirier GG, Lindahl T (1993). NAD⁺-dependent repair of damaged DNA by human cell extracts. *J. Biol. Chem.*, 268, 5480-5487.
- 229 Clark JB, Ferris GM, Pinder S (1971). Inhibition of nuclear NAD nucleoside and poly ADP-ribose polymerase activity from rat liver by nicotinamide and 5-methylnicotinamide. *Biochim. Biophys. Acta*, 238, 82-85.
- 230 Preiss J, Schlager R, Hilz H (1971). Specific inhibition of poly ADP-ribose polymerase by thymidine and nicotinamide in HeLa cells. *FEBS Lett.*, 19, 244-246.
- 231 Claycomb WC (1976). Poly(adenosine diphosphate ribose) polymerase activity and nicotinamide adenine dinucleotide in differentiating cardiac muscle. *Biochem. J.*, 154, 387-393.
- 232 Banasik M, Ueda K (1994). Inhibitors and activators of ADP-ribosylation reactions. *Mol. Cell. Biochem.*, 138, 185-197.
- 233 Shimoyama M, Kawai M, Nasu S, Shioji K, Hoshi Y, Ueda I (1975). Inhibition of adenosine 3',5'-monophosphate phosphodiesterase by nicotinamide and its homologues *in vitro*. *Physiol. Chem. & Physics*, 7, 125-132.
- 234 Morris NR, Reichard R, Fischer GA (1963). Studies concerning the inhibition of cellular reproduction by deoxyribonucleotides. II. Inhibition of the synthesis of deoxycytidine by thymidine deoxyadenosine and deoxyguanosine. *Biochim. Biophys. Acta*, 68, 93-99.
- 235 Johnson GS, D'Armiento M, Carchman RA (1974). N⁶-Substituted adenines induce cell elongation irrespective of the intracellular cyclic AMP levels. *Exp. Cell. Res.*, 85, 47-56.

- 236 Cleaver JE, Thomas GH (1969). Single strand interruptions in DNA and the effects of caffeine in Chinese hamster cells irradiated with ultraviolet light. *Biochem. Biophys. Res. Commun.*, **36**, 203-208.
- 237 Shall S (1975). Experimental manipulation of the specific activity of poly(ADP-ribose) polymerase. *J. Biochem. (Tokyo)*, **77**, 2p
- 238 Purnell MR, Whish JD (1980). Novel inhibitors of poly(ADP-ribose) synthetase. *Biochem. J.*, **185**, 775-777.
- 239 Cantoni O, Sestili P, Spadoni G, Balsamini C, Cucchiaroni L, Cattabeni F (1987). Analogues of benzamide containing a sulfur atom as poly(ADP-ribose) transferase inhibitors. *Biochem. Int.*, **15**, 329-337.
- 240 Rankin PW, Jacobson EL, Benjamin RC, Moss J, Jacobson MK (1989). Quantitative studies of inhibitors of ADP-ribosylation *in vitro* and *in vivo*. *J. Biol. Chem.*, **264**, 4312-4317.
- 241 Sestili P, Spadoni G, Balsamini C, Scovassi I, Cattabeni F, Duranti E, Cantoni O, Higgins D, Thomson C (1990). Structural requirements for inhibitors of poly(ADP-ribose) polymerase. *J. Cancer Res. Clin. Oncol.*, **116**, 615-622.
- 242 Banasik M, Komura H, Saito I, Abed NAN, Ueda K (1989). New inhibitors of poly (ADP-ribose) synthetase. In : *ADP-Ribose Transfer Reactions : Mechanisms and Biological Significance* (Jacobson MK, Jacobson EL, eds.), Springer Verlag, New York, 130-133.
- 243 Banasik M, Komura H, Shimoyama M, Ueda K (1992). Specific inhibitors of poly(ADP-ribose) synthetase and mono(ADP-ribosyl) transferase. *J. Biol. Chem.*, **267**, 1569-1575.
- 244 Li H, Goldstein BM (1992). Carboxamide group conformation in the nicotinamide and thiazole-4-carboxamide rings : implications for enzyme binding. *J. Med. Chem.*, **35**, 3560-3567.
- 245 Suto MJ, Turner WR, Arundel-Suto CM, Werbel LM, Sebolt-Leopold JS (1991). Dihydroisoquinolinones: the design and synthesis of a new series of potent inhibitors of poly(ADP-ribose) polymerase. *Anticancer Drug Design*, **7**, 101-117.
- 246 Arundel-Suto CM, Scavone SV, Turner WR, Suto MJ, Sebolt-Leopold JS (1991). Effects of PD 128763, a new potent inhibitor of poly(ADP-ribose) polymerase, on

X-ray-induced cellular recovery processes in Chinese hamster V79 cells. *Radiation Res.*, 126, 367-371.

- 247 Pivazyan AD, Birks EM, Wood TG, Lin T-S, Prusoff WH (1992). Inhibition of poly(ADP-ribose) polymerase activity by nucleoside analogues of thymidine. *Biochem. Pharm.*, 44, 947-953.
- 248 Bleasdale C, Calvert AH, Curtin NJ, Durkacz BW, Golding BT, Griffin RJ, Newell DR, Pemberton LC, Rhodes D (1994). Inhibitors of poly(adenine diphosphate ribose) polymerase to potentiate DNA reactive drugs. *Br. J. Cancer*, 69 (suppl. XXI) 16.
- 249 Griffin RJ, Pemberton LC, Rhodes D, Bleasdale C, Bowman K, Calvert AH, Curtin NJ, Durkacz BW, Newell DR, Porteous JK, Golding BT (1995). Novel potent inhibitors of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP). *Anticancer Drug Design*, 10, 507-514.
- 250 Ruf A, Ménissier de Murcia J, De Murcia GM, Schulz G (1996). Structure of the catalytic fragment of poly(ADP-ribose) polymerase from chicken. *Proc. Natl. Acad. Sci. USA*, 93, 7481-7485.
- 251 White AW (1996). The design of novel inhibitors of poly(ADP-ribose) polymerase to potentiate cytotoxic drugs. *PhD Thesis*, University of Newcastle upon Tyne.
- 252 Plescia S, Bajardi ML, Raffa D, Daidone G, Matera M, Caruso A, Amico-Roxas M (1986). Synthesis and pharmacological study of some 3-(pyrazol-5-yl)-quinazolin-4-[3H]-ones. *Eur. J. Med. Chem.*, 21, 291-295.
- 253 Dempcy RO, Skibo EB (1992). Rationale drug design of purine nucleoside phosphorylase inhibitors: design of 2-(2'-haloethyl) and 2-ethenyl substituted quinazolinone alkylating agents. *Bioorg. Med. Chem. Lett.*, 2, 1427-1434.
- 254 Peet NP, Baugh LE, Sunder S, Lewis JE, Matthews EH, Uberding EL, Shah DN (1986). 3-(1H)-Tetrazol-5-yl)-4-(3H)-quinazolinone sodium salt (MDL427): a new antiallergic agent. *J. Med. Chem.* 29, 2403-2409.
- 255 Allen EE, de Laszlo SE, Huang SX, Quagliato CS, Greenlee WJ, Chang RSL, Chen T-B, Faust KA, Lotti VJ (1993). Quinazolinones-1. Design and synthesis of

- potent quinazolinone-containing AT(1)-selective angiotensin-II receptor antagonists. *Bioorg. Med. Chem. Lett.*, **3**, 1293-1298.
- 256 Jackman AL, Taylor GA, Gibson W, Kimbell R, Brown M, Calvert AH, Judson IR, Hughes LR (1991). ICID1694, A quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumour cell growth in vitro and in vivo: A new agent for clinical study. *Cancer Res.*, **51**, 5579-5586.
- 257 Baudoin B, Ribeill Y, Vicker N (1993). A facile preparation of quinazolin-4-[3N]-ones from *o*-amido benzonitriles using sodium perborate. *Synth. Commun.*, **23**, 2833-2837.
- 258 Levin JI, Venkatesan AM, Chan PS, Baker JS, Francisco G, Bailey T, Vice G, Katocs A, Lai F, Coupet J (1994). 2,3,6-Substituted quinazolinones as angiotensin II receptor antagonists. *Bioorg. Med. Chem. Lett.*, **4**, 1135-1140.
- 259 Hilmy KMH, Mogensen J, Pedersen EB (1987). Phosphorus pentoxide in organic synthesis. XXX. New synthesis of 4(3*H*)-quinazolinones. *Acta Chem. Scand.*, **B41**, 467-468.
- 260 Takeuchi H, Hagiwara S, Eguchi S (1989). A new efficient synthesis of imidazolinones and quinazolinones by intramolecular aza-Wittig reaction. *Tetrahedron*, **45**, 6375-6386.
- 261 Akazome M, Kondo T, Watanabe Y (1993). Transition-metal complex-catalyzed reductive *N*-heterocyclization: Synthesis of 4(3*H*)-quinazolinone derivatives from *N*-(2-nitrobenzoyl).amides. *J. Org. Chem.*, **58**, 310-312.
- 262 Yoshida S, Aoyagi T, Harada S, Matsuda N, Ikeda T, Naganawa H, Hamada M, Takeuchi T (1991). Production of 2-methyl-4-[3*H*]-quinazolinone, an inhibitor of poly(ADP-ribose) synthetase by bacterium. *J. Antibiotics*, **44**, 111-112.
- 263 Pemberton LC (1994). Novel inhibitors of poly adenine diphosphate ribose polymerase to potentiate DNA reactive drugs. *PhD Thesis*, University of Newcastle upon Tyne.
- 264 Boulton S, Pemberton LC, Porteous JK, Curtin NJ, Griffin RG, Golding BT, Durkacz BW (1995). Potentiation of temozolomide cytotoxicity : a comparative study of the biological effects of poly(ADP-ribose) polymerase inhibitors. *Br. J. Cancer*, **72**, 849-856.

- 265 Ciereszko LS, Hanks LV (1954). Intermediates in the synthesis of carboxyl- C^{14} -labeled 3-hydroxyanthranilic acid. *J. Am. Chem. Soc.*, 76, 2500-2502.
- 266 Bhatt MV, Kulkarni SU (1983). Cleavage of ethers. *Synthesis*, 249-340.
- 267 Rappoport Z (editor). (1970). The chemistry of the cyano group, Chapter 3. Interscience, London.
- 268 Weijang Z, Koehler KF, Harris B, Skolnick P, Cook JM (1994). Synthesis of benzo-fused benzodiazepines employed as probes of the agonist pharmacophore of benzodiazepine receptors. *J. Med. Chem.*, 37, 745-757.
- 269 Pond SM, Tozer TN (1984). First-pass elimination. Basic concepts and clinical consequences. *Clin. Pharmacokinetics*, 9, 1-25.
- 270 Thomsen KF, Strøm F, Sforzini BV, Begtrup M, Mørk N (1994). Evaluation of phenyl carbamates of ethyl diamines as cyclisation-activated prodrug forms for protecting phenols against first-pass metabolism. *Int. J. Pharm.*, 112, 143-152.
- 271 Hausen J, Mørk N, Bundgaard H (1992). Phenyl carbamates of amino acids as prodrug forms for protecting phenols against first-pass metabolism. *Int. J. Pharm.*, 81, 253-261.
- 272 Dittert LW, Higuchi T (1963). Rates of hydrolysis of carbamate and carbonate esters in alkaline solution. *J. Pharm. Sci.*, 52, 852-857.
- 273 Bedford SB, Quarterman CP, Rathbone DL, Slack JA Griffin RJ, Stevens MFG (1996). Synthesis of water-soluble prodrugs of the cytotoxic agent Combretastatin A4. *Bioorg. Med. Chem. Lett.*, 6, 157-160.
- 274 Williams A, Douglas KT (1975). Elimination-addition mechanisms of acyl transfer reactions. *Chem. Rev.*, 75, 627-649.
- 275 Williams A, Al-Rawi H (1977). Elimination-addition mechanisms of acyl transfer reactions: The hydrolysis and synthesis of carbamates. *J. Amer. Chem. Soc.*, 99, 2671-2678.
- 276 Pettit GR, Temple Jnr. C, Narayanan VL, Varma R, Simpson MJ, Boyd MR, Rener GA, Bansal N (1995). Antineoplastic agents. 322. Synthesis of Combretastatin A-4 prodrugs. *Anticancer Drug Design*, 10, 299-309.
- 277 Mamber S, Mikkilineni AB, Pack ES, Rosser MP, Wong H, Ueda Y, Forenza S (1995). Tubulin polymerization by paclitaxel (taxol) phosphate prodrugs after

- metabolic activation with alkaline phosphatase. *J. Pharm. Exp. Therapeutics*, **274**, 877-883.
- 278 Saulnier MG, Langley DR, Kadow JF, Doyle TW (1994). Synthesis of etoposide phosphate, BMY-40481: A water-soluble clinically active prodrug of etoposide. *Bioorg. Med. Chem. Lett.*, **4**, 2567-2572.
- 279 Saulnier MG, Senter PD, Kadow JF (1990). *US Patent*, 4 904 768, Feb 27.
- 280 Kenner GW, Todd AR, Weymouth FJ (1952). Nucleotides. Part XVII. *N*-Chloroamides as reagents for the chlorination of diesters of phosphorous acid. A new synthesis of uridine-5' pyrophosphate. *J. Chem. Soc. (London)*, 3675-3681.
- 281 Wang MF (1992). The synthesis of carbohydrate analogues of inosital phosphates. *PhD Thesis*, University of Newcastle upon Tyne.
- 282 Atherton FR, Openshaw HT, Todd AR (1945). Studies on phosphorylation. Part I. Dibenzyl chlorophosphonate as a phosphorylating agent. *J. Chem. Soc. (London)*, 382-385.
- 283 Atherton FR, Howard HT, Todd AR (1948). Studies on phosphorylation. Part IV. Further studies on the use of dibenzyl chlorophosphonate and the examination of certain alternative phosphorylation methods. *J. Chem. Soc. (London)*, 1106-1111.
- 284 Silverberg LJ, Dillon JL, Vemishetti P (1996). A simple, rapid and efficient protocol for the selective phosphorylation of phenols with dibenzyl phosphite. *Tet. Lett.*, **37**, 771-774.
- 285 Atherton AR, Openshaw HT, Todd AR (1945). Studies on phosphorylation. Part II. The reaction of dialkyl phosphites with polyhalogen compounds in presence of bases. A new method for the phosphorylation of amines. *J. Chem. Soc. (London)*, 660-663.
- 286 King PE, Acheson RM (1949). The synthesis of benziminazoles from ortho-phenylene diamines and imino-ethers. *J. Chem. Soc.*, 1396-1400.
- 287 Thomas AP, Allott CP, Gibson KH, Major JS, Masek BB, Oldham AA, Ratcliffe AH, Roberts DA, Russell ST, Thomason DA (1992). New nonpeptide angiotensin II receptor antagonists. I. Synthesis, biological properties and structure-activity relationships of 2-alkyl benzimidazole derivatives. *J. Med. Chem.*, **35**, 877-885.

- 288 Kubo K, Kohara Y, Imamiya E, Sugiura Y, Inada Y, Furkawa Y, Nishikawa K, Naka T (1993). Nonpeptide angiotensin II receptor antagonists. Synthesis and biological activity of benzimidazole carboxylic acids. *J. Med. Chem.*, 36, 2182-2195.
- 289 Iemura R, Kawashima T, Fukuda T, Ito K, Tsukamoto G (1986). Synthesis of 2-(4-substituted-1-piperazinyl) benzimidazoles as H-1-antihistaminic agents. *J. Med. Chem.*, 29, 1178-1183.
- 290 Kim JS, Sun Q, Gatto B, Yu C, Lui A, Lui LF, LaVoie EJ (1996). Structure-activity relationships of benzimidazoles and related heterocycles as topoisomerase I poisons. *Bioorg. Med. Chem.*, 4, 621-630.
- 291 Lácová M, Abliami S, Perjéssy A, Králová K, Chovancová J (1993). Preparation, infrared spectra and growth-regulating activity of 2-substituted benzimidazoles. *Chem. Papers*, 47, 393-400.
- 292 Vlaovic D, Canadanovic-Brunet J, Balaz J, Juranic I, Djokovic D, Mackenzie K (1992). Syntheses, antimicrobial and antifungal activities of some new benzimidazoles. *Biosci. Biotech. Biochem.*, 56, 199-206.
- 293 Phillips MA (1928). The formation of 2-methyl benzimidazoles. *J. Chem. Soc.*, 13, 172-177.
- 294 Phillips MA (1928). The formation of 2-substituted benzimidazoles. *J. Chem. Soc.*, 13, 2393-2399.
- 295 Alcalde E, Dinarés I, Pérez-García L, Roa T (1992). An advantageous synthesis of 2-substituted benzimidazoles using polyphosphoric acid. 2-(Pyridyl)-1*H*-benzimidazoles, 1-Alkyl-(1*H*-benzimidazol-2-yl) pyridinium salts, their homologues and vinylogues. *Synthesis*, 395-398.
- 296 Crotti C, Cenini S, Ragaini F, Porta F (1992). Ruthenium carbonyl-catalysed deoxygenation by carbon monoxide of *o*-substituted nitrobenzenes. Synthesis of benzimidazoles. *J. Mol. Catalysis*, 72, 283-298.
- 297 Katritzky AR, Yang B, Abonia R, Insuasty B (1996). Synthesis of 1,2-disubstituted benzimidazoles by the photolysis of imidoyltriazoles. *J. Chem. Res. (S)*, 540-541.

- 298 DeLuca MR, Kerwin SM (1997). The *para*-toluenesulfonic acid-promoted synthesis of 2-substituted benzoxazoles and benzimidazoles from diacylated precursors. *Tetrahedron*, 53, 457-464.
- 299 Popp FD (1975). The chemistry of isatin. *Adv. Heterocyclic Chem.*, 18, 1-58.
- 300 Sandmeyer T (1919). Über Isonitrosoacetanilide und deren Kondensation zu Isatinen. *Helv. Chim. Acta*, 2, 234-242.
- 301 Buchman ER, McCloskey CM, Seneker JA (1947). 8-Nitrocinchoninic acids and related substances. *J. Amer. Chem. Soc.*, 69, 380-384.
- 302 Marvel CS, Hiers GS (1941). Isatin. *Org. Synth. Coll. Vol.*, 1, 327-330.
- 303 Denny WA, Rewcastle GW, Baguley BC (1990). Potential antitumor agents. 59. Structure-activity relationships for 2-phenyl benzimidazole-4-carboxamides, a new class of "minimal" DNA-intercalating agents which may not act via topoisomerase II. *J. Med. Chem.*, 33, 814-819.
- 304 Weidenhagen R, Weeden U (1938). Eine neue Synthese von Benzimidazol-Derivaten. *Chem. Ber.*, 69B, 2263-2272.
- 305 Mathias LJ (1979). Esterification and alkylation reactions employing isoureas. *Synthesis*, 561-576.
- 306 Halldorsson H, Gray DA, Shall S (1978). Poly(ADP-ribose) polymerase activity in nucleotide permeable cells. *FEBS Lett.*, 85, 349-352.
- 307 Grube K, Küpper JH, Bürkle A (1991). Direct stimulation of poly(ADP-ribose) polymerase in permeabilised cells by double-stranded oligomers. *Anal. Biochem.*, 193, 236-239.
- 308 Griffin RJ, Srinivasan S, White AW, Bowman, K, Calvert AH, Curtin NJ, Newell DR, Golding BT (1996). Novel benzimidazole and quinazolinone inhibitors of the DNA repair enzyme poly(ADP-ribose) polymerase. *Pharm. Sci.*, 2, 43-47.
- 309 Stevens MFG, Hickman JA, Stone R, Gibson NW, Baig GU, Lunt E, Newton CG (1984). Antitumor imidazotetrazines. I. Synthesis and chemistry of 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one, a novel broad-spectrum antitumor agent. *J. Med. Chem.*, 27, 196-201.
- 310 Stevens MFG, Newlands ES (1993). From triazines and triazenes to temozolomide. *Eur. J. Cancer*, 29A, 1045-1047.

- 311 Bowman K, Calvert AH, Curtin NJ, Golding BT, Griffin RJ, Newell DR, Srinivasan S, White AW (1996) Effect of novel PARP inhibitors on the cytotoxicity of anticancer agents. *Br. J. Cancer*, 73, Suppl. 26, 13.
- 312 Pratt WB, Ruddon RW, Ensminger WD, Maybaum J (1994). The anticancer drugs (2nd edn.), Oxford University Press, Inc.
- 313 Stryer L (1988). Biochemistry (3rd edn.), Freeman & Co., New York.
- 314 Perrin DD, Armarego WLF (1988). Purification of laboratory chemicals (3rd edn.), Pergamon Press, Ltd., Oxford, New York, Seoul, Tokyo.